EXPERIMENTAL SALMONELLA INFECTIONS IN AUSTRALIAN COCKROACHES

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In a previous paper the occurrence of naturally infected cockroaches during an epidemic of salmonellosis in Brisbane was recorded. *Salmonella bovis-morbificans* was found in *Periplaneta americana*,[1] and *S. typhi-murium* in *Nauphoeta cinerea*. As cockroaches abound in Brisbane, live many months, and move rather freely from place to place, it seemed desirable to determine whether they could be infected at all readily, and for how long they would retain an infection. There were several instances recorded in the literature of cockroaches being infected with bacteria experimentally (e.g. with *V. cholerae* by Barber, 1914, and Toda, 1923; with *M. tuberculosis* and *M. leprae* by Macfie, 1922; with *M. tuberculosis* and staphylococci by Morrel, 1911; with several pathogenic species by Tejera, 1926), but few experiments were carried for more than three days, and none seemed to demonstrate that the organisms did more than pass alive through the intestinal tract of the insects. We have not seen the papers by Cao and Longfellow, which have been reviewed by Steinhaus (1946). There appeared, however, to be a fairly wide field of experimental work open to us.

METHODS.

The method of controlling and maintaining the cockroaches was adapted from that described by Wedberg and Clarke (1947). At first all stages were used, but adults proved easier to mount and thrived better under restraint than immatures, and were therefore used in the experiments reported here. Females were more satisfactory than males. The cockroaches were first quietened by placing them in the freezing chamber of the refrigerator for five to ten minutes. They were then mounted on blocks of hard paraffin, about an inch cube, by placing the cockroach's back on the side of the block, which had been flamed for a second. Their wings were fixed more securely by running melted paraffin around the edges, and the wing-tips were clipped just shorter than the abdomen. If the antennae were cut short (between a half and one inch long), the cockroaches appeared to settle down better, as they could not feel the walls of the jar and struggle to reach them. The paraffin blocks were securely attached to a cork, either by a piece of wire about two inches long, or directly by melted paraffin, and set up in glass dressing jars 6 inches wide by 6 inches high. A petri dish containing about 10 ml. tetrathionate broth was placed beneath each cockroach or group to catch the droppings.

[1] Probably included some *P. ignota* Shaw, which we had not then recognized as distinct from *P. americana*.

The top of the jar was covered with muslin, and a high humidity was maintained and evaporation of the broth retarded by keeping moist blotting paper at the bottom of the jar. Every morning each cockroach was fed on a sterile solution of sucrose by means of a pipette. After a day or two, when they had quietened down, solids were added to their diet by giving them small pieces of bread moistened in the sucrose solution and impaled on the end of a sterile platinum needle. They would first suck off the solution, and then devour the bread. Apple was also given from time to time by a similar technique, and was readily accepted. After a little practice, it was soon easy to judge the amount each cockroach could consume.

Cockroaches lived for several weeks under these conditions. Of the seven uninfected controls used in the experiments, one was killed after being mounted for 23 days, four died after 16, 39, 50 and 60 days respectively, and two are still alive after 89 and 111 days. They remained quite active throughout, insofar as their restrained position allowed them. The only abnormality encountered was an occasional case of extreme, acute, gaseous distention of the stomach, due, we suspect, to too much sucrose. Some were set up in pairs as a counter-measure against loss. Controls were kept in all experiments as a check on cross-infection.

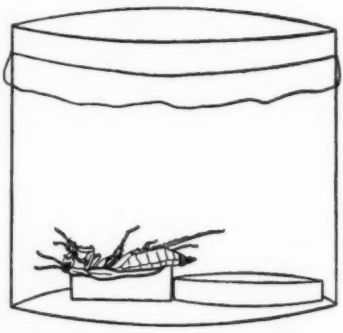

Fig. 1. Diagram of arrangement to control infected cockroaches.

Faeces were passed irregularly, at first almost daily, later with variable gaps, in one instance of 11 days. For 38 cockroaches under observation for a summated period of 473 days, the mean interval between defaecations was 2·5 days. The dishes of T.T. broth were changed daily. Any faeces present were emulsified with a swab-stick, the broth was incubated at 37° C. for 24 hours, plated on "Difco" S.S. agar, and resulting colonies tested for biochemical reactions and group agglutinogens.

At least three negative specimens were obtained from each cockroach before Salmonellas were fed to it. Sucrose broth cultures 18–24 hours old were used. At first, each insect was given one or two drops of culture from a fine pipette, later two or three loopfuls from a platinum loop about 1·5 mm. in diameter; it was always observed to suck up the whole of the dose. Normally only one dose was given, but multiple feeds were also tried in an endeavour to increase infection rates—five doses on successive days, three on successive days, three spread over five days; the results were poor. Starving the cockroaches for 48 hours after feeding a single dose of culture to them was more successful.

In addition to the faecal cultures made during the pre- and post-infection periods, separate cultures were made of the fore, mid and hind gut of each cockroach when it died or was killed at the end of its period of observation.

RESULTS OF FEEDING INDIVIDUAL COCKROACHES.

Six species of cockroaches occur not uncommonly in houses in Brisbane, namely: *Periplaneta australasiae* (Fabr.), *P. americana* (Linn.), *P. ignota* (Shaw), *Nauphoeta cinerea* (Oliv.), *Blatella germanica* (Linn.), and *Supella supellectilium* (Serv.).[2]

[2] There are specimens of *Blatta orientalis* Linn., from houses in Adelaide, in the Shaw collection in the Queensland Museum, but this species has not so far been found in Brisbane.

We used those that were most readily available. We also had ten species of Salmonella, which had been isolated during a recent outbreak of gastro-enteritis, and we used five of these. Altogether, 49 cockroaches were used in these series.

TABLE 1.

Individual infections.

Species of cockroach.	Species of Salmonella.				
	bovis-morbificans.	*typhi-murium.*	*adelaide.*	*derby.*	Totals.
Periplaneta australasiae	9 of 11	2 of 3	3 of 4	2 of 3	16 of 21
P. ignota	2+ of 4		1+ of 2		3 of 6
Supella supellectilium	1 of 1	2 of 2	2 of 2	3 of 3	8 of 8
Nauphoeta cinerea	1+ of 2	1+ of 3	2 of 2	3 of 4	7 of 11
Totals:	13 of 18	5 of 8	8 of 10	8 of 10	34 of 46

+ Indicates infection of pairs, in which it could not be distinguished whether both or only one of the pair was infected.

In addition, 3 of 3 *P. australasiae* became infected with *S. kottbus*.

The general results obtained are set out in Table 1. Salmonellas were isolated, either from the faeces during life or the gut *post mortem*, from about 75 p.c. of the cockroaches tested, and all species of cockroach used became infected with all species of Salmonella offered to them. The numbers are too small for statistical analysis, but there was no indication that different species of cockroach were more susceptible than others, or that different Salmonellas were more infective than others.

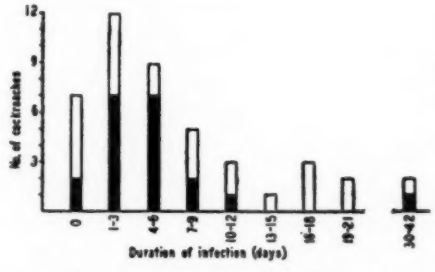

Fig. 2. Histogram of the duration of infection in 49 cockroaches. Plain, totals; black, deaths.

Duration of Infection.

Recovery of Salmonellas in the faeces was distinctly capricious, as may be seen from Table 2, in which the histories of representative infections are set out in detail. The reasons for the irregularities are not clear; they could be due to unreliability of the cultural method, but an analysis of the tetrathionate broth-SS plate technique on a large series of infant faeces indicated a reliability of the order of 60–80 p.c. in our hands. It seems more likely that infections became established in the cockroaches, and that excretion of the organisms in detectable numbers was intermittent.

Of 44 cockroaches which could be followed individually, 7 showed no evidence of infection whatever, either in the faeces (24 cultures) or *post mortem* (21 cultures), 22 either died or lost their infections within a week, and 15 remained infected for 8–42 days. We feel that

TABLE 2.

Records of some individual infections.

No.	Species of cockroach.	Species of Salmonella.	Days from last dose of Salmonella culture.																												
						0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
G	*P. ignota*	*bovis-morbificans*				.	—		—				—	—		P.M. +													
U	*N. cinerea*	*typhi-murium*				.		+	—				—			—		—				P.M. +							
AY	*N. cinerea*	*derby*				.		+	+	+		+			—						—		—			P.M. +			
AV	*P. australasiae*	*derby*				.	+	+	+		—			—	—	—		P.M. —											
AF	*P. australasiae*	*bovis-morbificans*	.	.	. +	. +	. —	+		+	—	—			--			P.M. +											
AI	*P. australasiae*	*bovis-morbificans*	.		. —	—	. —	—		—	—	P.M. —																	
BG	*N. cinerea*	*adelaide*				.		+		+	+	+	+		+		+	+	+	+	+		—		+		+	+	
						24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42					
						+	+		+		+			+		+				+	+	—	P.M. +					

. Indicates days on which Salmonella culture was fed to the cockroach.

there is a reasonable presumption that the infection did not simply pass through the intestine, but actually became established in at least the third group. The longest infections encountered were:

42 days in an *N. cinerea* infected with *S. adelaide.*
30 days in an *S. supellectilium* infected with *S. derby.*
20 days in an *N. cinerea* infected with *S. derby.*
19 days in an *P. ignota* infected with *S. bovis-morbificans.*
18 days in an *P. australasiae* infected with *S. kottbus.*
16 days in an *N. cinerea* infected with *S. typhi-murium.*

The data are set out more fully in Fig. 2.

Effects of Infection on the Host.

The infected cockroaches showed no obvious change in general activity, appetite, or number of stools produced. Nevertheless, there was a significantly higher mortality during the first week of infection than later (Fig. 2; $\chi^2 = 5{\cdot}7$ $P = {\cdot}02$). Also, the mortality in the infected cockroaches was higher than in the controls. Eighteen of 49 given Salmonellas died within 14 days as compared with 0 of 7 controls. The controls were set up primarily as a check on possible cross-infection and the possibility of a difference in mortality was not suspected at the time; their numbers are consequently not large enough for statistical treatment.

INFECTIONS IN COLONIES.

Our information from laboratory colonies of cockroaches is meagre, but may be recorded for the sake of completeness.

One colony, AQ, of adult and immature *N. cinerea,* living under natural though confined conditions, was offered a sucrose-broth culture of *S. typhi-murium* in a petri dish for 48 hours and again for 48 hours three days later. Except that no other fluid was available, this was considered to simulate reasonably the opportunities cockroaches would have in a ward sink where infected napkins were washed. The colony was sampled by removing 3–5 immatures and adults at intervals, and sowing the entire immatures and the intestines of the adults into tetrathionate broth. All samples made on the 3rd, 15th, and 25th day after the culture was first offered were *positive,* but the final sample on the 32nd day was negative. In addition, *S. typhi-murium* was isolated from a filter-paper, which formed part of the coverage in the jar and which had become soiled with cockroach faeces, and from SS-agar plates, on which some of the cockroaches were allowed to walk.

The inferences from this experiment were so obvious that it did not seem necessary to repeat it.

Four colonies, two of *P. australasiae* and two of *N. cinerea,* were set up in an endeavour to determine whether an infection, once introduced, would spread in a colony of cockroaches. One infected individual was introduced into each of the first three colonies (*S. bovis-morbificans* in one, *S. typhi-murium* in the other two). Except for one of the originally infected insects, no subsequent evidence of infection was found. Four cockroaches from AQ (above) were introduced into the fourth colony. These individuals were subsequently found still to be infected up to the 15th day, but no infections were detected in any other member of the colony.

DISCUSSION.

The purpose of the work was to determine whether cockroaches would act as vectors, or even as temporary reservoirs, of Salmonella infections, and we believe that that purpose has been sufficiently fulfilled. It has been shown that they do acquire infections readily, and that about 15 p.c. become "chronic carriers," in that they retain the infection for more than a fortnight. They appear to be intermittent excretors of the organisms, but one insect produced 22 infected stools in 42 days, another 12 in 19 days, and altogether 83 positive faecal cultures were obtained during the experiments, apart from the infected colony.[3] As cockroaches feed readily both on faeces and food, their capacity to spread salmonellosis is thus by no means negligible.

That these considerations are not purely theoretical is indicated by our experience during a recent outbreak of gastro-enteritis in Brisbane. We isolated Salmonellas from ward sinks and brushes, to which cockroaches would have ready access, and we isolated them from cockroaches captured in the wards (Mackerras and Mackerras, 1948). They would not be likely to infect the food of infants, but they could easily have contaminated the food of older children and adults and thus contributed to the vicious circle of infection.

Their exact rôle in any particular case can only be determined by detailed epidemiological studies, but we believe that we have adduced sufficient evidence to justify intensive control measures in any area where both cockroaches and Salmonella infections are prevalent.

SUMMARY.

Four species of cockroaches have been infected experimentally with five species of Salmonella, about 75 p.c. of insects tested becoming infected and 15 p.c. retaining their infections for more than a fortnight. Viable Salmonellas were excreted in the faeces up to 40 days from infection.

Acknowledgment. We are grateful to Miss Nancy Atkinson of the Institute of Medical and Veterinary Science, Adelaide, for identifying the strains of Salmonella used.

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[3] In a more recent series, fluid regurgitated from the mouth has also been examined. Positive cultures were obtained between the second and fifth day after infection.

THE REACTION OF PERFUSION FLUIDS IN LONG LASTING EXPERIMENTS

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Rules for preparing the various physiological salt solutions ensure that their initial pH is of physiological order. In experiments on surviving organs it is equally important to keep the pH constant for any length of time. The pH of the common perfusion fluids, however, is liable to change to a far greater extent than might be expected. One particular point of importance is that oxygenation with air or oxygen, still being frequently practised, tends to raise the pH of insufficiently buffered solutions.

In the following some observations dealing with this matter will be presented. The immediate purpose of this study was to overcome certain difficulties which were encountered in experiments on surviving arteries. Such preparations may exhibit spontaneous rhythmic contractions; but there are discrepancies in the statements as to the best conditions for this rhythmic activity. According to some observers (Rothlin, 1920; Ducret, 1931) oxygenation of the salt solutions is necessary for the initiation of rhythmic contractions; others found oxygen either unnecessary (Apitz, 1920; Wachholder, 1921) or even unfavourable (Meyer, 1913).

Since the oxygen requirement in general of the plain muscle is relatively low, oxygenation might have proved effective in these experiments not only by providing oxygen but by some secondary action. One side effect influencing the phenomenon could have been a change in the reaction of the salt solution. As most experiments on surviving vessels have been made with unbuffered solutions oxygenated in the customary way it was desirable to find out more accurately what the pH conditions in such experiments, particularly if extending over several hours, actually are. Our own experiments in this direction extend and confirm some observations by others. The relevant facts being little known, a summary of our findings with reference to the older observations might be of some use for those working with oxygenated salt solutions.

That escape of CO_2 from salt solutions must unavoidably lead to a change in the reaction has been a matter of concern for many workers ever since the reaction was recognized as a fundamental environmental factor. To prevent this change two measures became established: (a) use of phosphate buffers, and (b) continuous CO_2 supply together with oxygenation. The first method, although effective, within limits, in maintaining the pH, is unphysiological in that it does not provide the bicarbonate buffer system. As will be seen later, there is reason to believe that this system is of physiological importance apart from its function in cH regulation. The practice of adding between three and six volumes per cent. CO_2 to air or oxygen is not

[1] This work was carried out with the aid of the National Health and Medical Research Council.

always carried out in such a way as to secure optimal physiological conditions. To clarify certain points concerning CO_2 supply to salt solutions we have carried out experiments, the results of which may be found useful for work with artificial media.

METHODS.

All pH estimations were made with Cambridge pH meters, using a Morton glass electrode system. The accuracy of the estimations was well within $\pm 0{\cdot}02$ pH. External electrical disturbances could be completely eliminated. Most observations were carried out at body temperature. To do this in a convenient way the pH meter, stock solutions, distilled water and the fluids under test were placed in a hot room kept at 37° to 38° C. The gas mixtures to which the solutions were exposed were stored (gasmeters, cylinders) outside; these gases before reaching the fluids were well pre-heated by passing through a long system of glass tubes in the hot room. The temperature of the test fluids decreased in spite of this measure by 1° C. or more in the course of several hours, but this did not affect the essential results. The gases were bubbled through a water column in order to saturate them with water vapour.

About 250 ml. of the fluid to be tested were placed in a beaker or flask with oxygen or a CO_2 mixture bubbling through it at a steady rate. At due intervals samples were taken and pH estimations made without delay. Within the few seconds required for this operation the pH did not change to any noticeable degree. The temperature was measured at the same time.

The gas mixtures used were made up and checked by means of gas analysis. Commercial oxygen-CO_2 mixtures (carbogen) for medical use were found unreliable in their composition. All solutions were made up with glass distilled water.

RESULTS.

pH Changes of Tyrode's and Fleisch's Solution with and without Oxygenation.

Sollmann *et al.* (1928) have carried out a study on Locke's solution ($NaCl = 0{\cdot}92$, $KCl = 0{\cdot}042$, $CaCl_2 \cdot 2H_2O = 0{\cdot}024$) containing varying amounts of $NaHCO_3$ and Na_2HPO_4. They found colorimetrically that with bicarbonate concentrations above 0·01 p.c. and without phosphate the pH of these fluids under vigorous aeration (30 ml. of air per minute for 50 ml. of the solution) increased considerably within a few hours; monosodium phosphate addition accelerated this alkalinization. Certain bicarbonate and diphosphate mixtures were found to stabilize the pH. The presence of 0·1 p.c. glucose in the solution did not affect the pH. The authors remark that the results obtained applied strictly to the particular solution investigated and may not be directly transferred to others, especially since variations in the calcium content may influence the reaction of the buffer mixtures. Attention was also paid to the manner in which living tissues (pieces of intestine) immersed in smaller volumes of such solutions may influence the reaction by producing CO_2 and fixed acids.

Some observations in this line have also been made on Fleisch's solution (Chopra and Ghosh, 1925; Sollmann *et al*, 1928). This solution, buffered by the phosphate and bicarbonate buffer systems, is claimed to be very stable; it has recently been used by Swiss authors for experiments on excised pieces of arteries (Monnier, 1943, 1944; Bürgi, 1944). Only a small rise of pH in several hours was observed, but further data concerning this fluid seemed desirable. Sollmann *et al.* pointed out that the relatively high phosphate content of Fleisch's solution causes alterations in Ca and probably also Mg ions as indicated by the occurrence of precipitations.

A few observations by Chopra and Ghosh (1925) refer to Tyrode's solution, but no figures were reported.

In order to obtain more information concerning this matter, the pH changes that take place in Tyrode and Fleisch's solutions at body temperature and under ordinary experimental conditions were studied. The solutions under observation were either left standing or oxygen was passed through them at a rate of 10 to 15 ml. of gas per minute for about 250 ml. of solution.

The composition of Tyrode's solution is: $NaCl = 0{\cdot}8$, $KCl = 0{\cdot}02$, $CaCl_2 = 0{\cdot}02$, $MgCl_2 = 0{\cdot}01$, $NaH_2PO_4 = 0{\cdot}005$, $NaHCO_3 = 0{\cdot}1$, glucose $= 0{\cdot}1$, distilled water to 100 ml. To avoid precipitations the ingredients should be dissolved in the above order (Carrel, 1938). The solution can also be made satisfactorily from two stock solutions (Hirz, 1913; Dittler, 1930); Landois, 1932). Solution 1, 20 p.c. NaCl, 0·5 p.c. KCl, 0·5 p.c. $CaCl_2$, and 0·25 p.c. $MgCl_2$; Solution 2, 5 p.c. $NaHCO_3$ and 0·25 p.c. NaH_2PO_4. Glucose is added finally. In our experiments only analytical reagents were used; stock Solution 1 was diluted and solid $NaHCO_3$ and NaH_2PO_4 added later. In most experiments glucose was omitted since it was found to have no influence on the reaction. The pH of the fresh Tyrode solution is 7·5 to 7·8 and can, if desired, be adjusted either by adding diluted HCl or by passing CO_2 through the fluid in order to convert any carbonate present into bicarbonate; this latter procedure may also be applied to a solution of $NaHCO_3$ before it is added to the solution. The effect on the pH of higher bicarbonate concentrations was also examined, with regard to the finding that higher bicarbonate contents favour the above-mentioned rhythmic activity of arteries (Apitz, 1920; Wachholder, 1921).

Fleisch's solution (Fleisch, 1922) has the following composition: $NaCl = 1{\cdot}0$, $KCl = 0{\cdot}05$, $CaCl_2 = 0{\cdot}03$, $MgCl_2 = 0{\cdot}01$, $H_3PO_4 = 0{\cdot}049$, $Na_2CO_3 = 0{\cdot}05$, distilled water to 100 ml. It is conveniently prepared from a durable stock solution ($NaCl = 15{\cdot}0$, $KCl = 0{\cdot}5$, $CaCl_2 = 0{\cdot}3$, $MgCl_2 = 0{\cdot}7$, N. $H_3PO_4 = 5$ ml., H_2O to 50 ml.) by mixing 50 ml. of it with 1,000 ml. distilled water and finally adding 5 ml. N. Na_2CO_3. The pH of this solution is 7·5. By interaction of H_3PO_4 and Na_2CO_3 the two buffer systems: H_2CO_3-$NaHCO_3$ and NaH_2PO_4-Na_2HPO_4 are formed with a resulting CO_2 concentration of 0·44 volume p.c. in the solution; CO_2 will escape, particularly on aeration, but this and the resulting alkalinization could be prevented by passing through the solution gas mixture containing 0·4 p.c. CO_2.

To meet special experimental requirements several modifications of this solution have been suggested, e.g. one with a lower bicarbonate content, by replacing a proportion of Na_2CO_3 with NaOH at the ratio: 1 ml. N. $Na_2CO_3 = 0{\cdot}535$ ml. N. NaOH. Another modification may contain three to four times more carbonate to make its concentration equal to that of blood. An increase of the phosphate concentration, for the purpose of obtaining higher buffering power has also been considered. The composition of Fleisch's solution is in many respects favourable, but is unphysiological in that the phosphate concentration is higher and the carbonate concentration far lower (three to four times) than the corresponding concentration in the plasma. Whether the pH can really be regarded as sufficiently stable for long-lasting experiments will be seen later. It is clear, moreover, that a higher carbonate content would deprive the solution of its relative pH stability, but on the other hand, a lower carbonate content or a higher phosphate concentration would create still more unphysiological conditions.

The results of our experiments were exemplified in the Figs. 1 and 2. The results can be summarized as follows:

(*a*) *Tyrode's solution.* The pH of this fluid is extremely unstable. At body temperature and with oxygenation at the rate stated the pH rises from the initially adjusted figures (7·3 to 7·5) in about one hour to 8·0 and in six hours

to 8·3; without oxygenation this rise is slower, but leads also to values above 8·0. These findings supplement and confirm previous observations by Chopra and Ghosh (1925) and are in agreement with the results of Sollman *et al.* (1928) concerning Locke's solution. As can be expected, the pH is higher with higher bicarbonate contents.

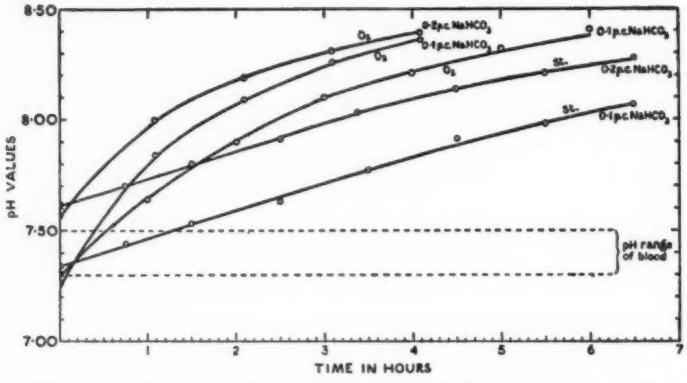

Fig. 1. pH changes of Tyrode's solution at body temperature. Initial pH adjusted by treatment for several minutes with 6 p.c. CO_2 mixture. O_2 = solutions oxygenated. St = solutions left standing.

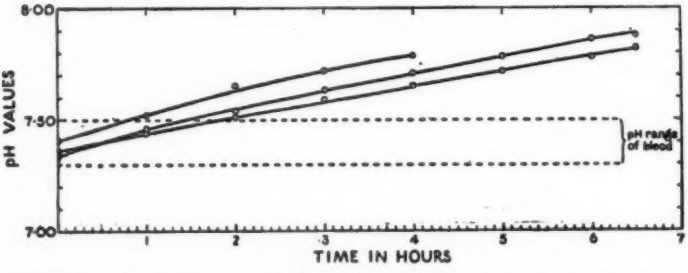

Fig. 2. pH changes of Fleisch's solutions at body temperature. Solutions oxygenated.

(*b*) *Fleisch's solution.* The greater stability of this solution is illustrated in Fig. 2. With oxygenation at body temperature the initial pH values of between 7·3 and 7·5 increase in several hours slowly to 7·6 to 7·8. This is in agreement with the results of Chopra and Ghosh (1925). Considering the fact that in normal life the cH of the blood is kept constant within about ±25 p.c. (cH mean value: $0{\cdot}4\times10^{-7}$), it seems desirable that in experiments with artificial media the cH should not be allowed to vary to a greater extent than

this, or, in other words, pH changes should not exceed 0·2. It appears that Fleisch's solution does not meet this requirement in experiments of long duration, unless here, too, special measures, such as outlined before, are taken.

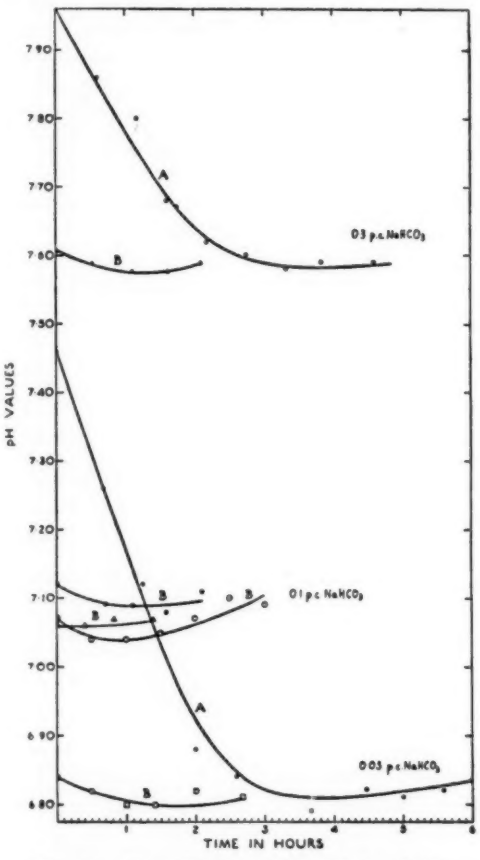

Fig. 3. pH equilibrium of Tyrode's solutions at different ratios of CO_2 and $NaHCO_3$ concentrations. a = solutions without preliminary pH adjustment; b = solutions first exposed for several minutes to vigorous aeration with CO_2 mixture; final pH value reached in a short time.

Adjustment of pH by Means of Variation of CO_2 and $NaHCO_3$ Concentrations.

Experiments were made to ascertain the accuracy of this method of pH control in ordinary work with salt solutions, particularly for longer periods. The pH values can be calculated according to Hasselbalch–Henderson's equation (Hasselbalch, 1917); but it depends entirely on the special experimental conditions whether this method of pH regulation will satisfy physiological requirements.

Only two CO_2 concentrations, namely 3·0 and 6·0 volume p.c. in oxygen were examined. All experiments were made at body temperature. The gas mixtures (accurate within ± 3 p.c. of their CO_2 values) were passed at steady rate through the fluids (250 ml.) and pH estimations were made until the values became constant for at least one hour. The equilibrium thus indicated could be established more quickly by first exposing the fluids to a vigorous aeration with the gas mixture for several minutes. The solutions were not covered with liquid paraffin as has been recommended occasionally to facilitate the establishment of an equilibrium.

The results obtained are illustrated in the Figs. 3 and 4 and can be stated

as follows: with CO_2 of a certain concentration passing at a steady rate through Tyrode's solution the pH can be adjusted with a high degree of accuracy by varying the bicarbonate content of the solution; pH differences of 0·1 to 0·2 can be produced. With both of the CO_2 concentrations tested the biological pH range (7·0 to 7·8) can be covered; for the pH range of blood (7·3 to 7·5) the required bicarbonate concentrations are between 0·1 and 0·25 p.c. The pH once established can be kept constant well within 0·1 for considerable periods of time.

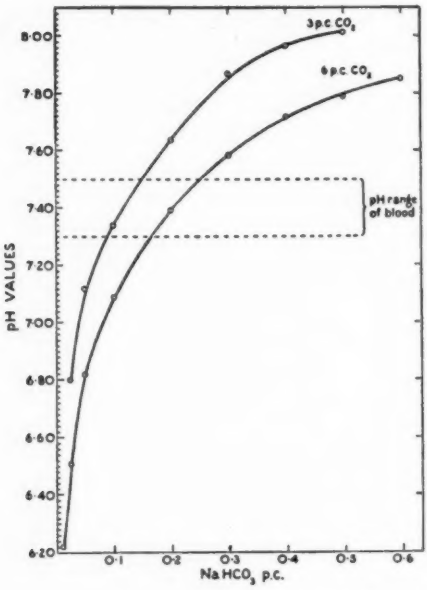

Fig. 4. pH values of Tyrode's solutions of different $NaHCO_3$ contents, aerated at constant rate with 3 p.c. and 6 p.c. CO_2-oxygen mixtures.

As regards the CO_2 tension in salt solutions it is clear that it should preferably be equal to the ordinary CO_2 tension in the plasma under normal resting conditions (40–45 mm. Hg.). The CO_2 tension (at 760 mm. atmospheric pressure and at 38° C.) in CO_2 mixtures is: $[(760–48)/100] \times CO_2$ volume p.c. (48 = correction for water vapour tension). Table 1 shows the CO_2 tensions so calculated for mixtures containing 2–10 p.c. CO_2. It can be seen that CO_2 concentrations of 5–6 volumes p.c. fulfil best the above requirement. The CO_2 tensions obtained with other CO_2 mixtures represent either extreme values still compatible with the normal acid-base balance (Van Slyke, 1921) or abnormal values (below 20 mm. and above 60 mm. Hg.).

TABLE 1.

CO_2 p.c.	CO_2 tensions. mm. Hg.	CO_2 p.c.	CO_2 tensions. mm. Hg.
10	71·0	5	35·5
8	57·0	4	28·5
7	50·0	3	21·0
6	43·5	2	14·0

The concentrations of dissolved CO_2 in the Tyrode's solution may also be considered. They can be calculated from the absorption coefficient. Assuming, on the basis of observation by Van Slyke *et al.* (1933), an absorption coefficient of CO_2 in Tyrode solution at 38° C. of approximately 0·5, the volume percentage of absorbed CO_2 in Tyrode's solution for 3 and 6

p.c. CO_2 is approximately 1·4 and 2·8 respectively. The figures for different bicarbonate contents will vary slightly. Free CO_2 in plasma amounts to 1·6 to 1·8 volumes p.c. and in whole blood to 2·4 to 2·7 volumes per cent.

DISCUSSION.

In the experiments described substantial evidence confirming some analogous older work has been obtained. Tyrode's solution, like other insufficiently buffered salt solutions, becomes spontaneously more and more alkaline, the pH increasing from initial values of around 7·5 to as much as 8·3, i.e. beyond the pH range compatible with life. Thus, the instability of Tyrode's solution is far greater than is commonly known. Hence, experiments with such solutions, unless only of shorter duration, are made at an unphysiological pH range. This refers, in particular, to the older experiments on surviving vessels suspended in aerated salt solutions (MacWilliam, 1902; de Bonis and Suzanna, 1909; Cow, 1911; Campbell, 1911; Barbour, 1912; Hooker, 1912; Wachholder, 1921). Some of these authors actually stress the point, that a more alkaline reaction was favourable for the elicitation of rhythmic activity. As this case shows, an alkaline reaction, therefore, need not necessarily, at least for some time, unfavourably affect the particular function or phenomenon under observation. The method of standardizing pituitary extracts by studying the responses of the guinea-pig uterus suspended in oxygenated, unbuffered, Ringer-Locke solution (Burn and Dale, 1921) may be quoted as another example in which the unavoidable alkalinization of the solution applied does not matter so far as the special object of this experiment is concerned. However, when a constant and physiological reaction is desirable for the purpose of the experiment, unbuffered solutions, Tyrode's or otherwise, although still in use, are obviously quite inadequate.

As stated already, the use of phosphate buffers, usually combined with reduction or complete elimination of carbonate (e.g., Fleisch, 1922; Iwai, 1924), whilst suitable for stabilizing pH, must be regarded as unphysiological, since it does not procure the bicarbonate buffer system. In addition, phosphates, at least in higher concentrations, may have some untoward effect by causing chemical changes in salt solutions and perhaps also through the phosphate ions *per se* (Sollman *et al.*, 1928; Barlow, 1929).

The cH of blood being essentially determined by the ratio $[H_2CO_3]$: $[BHCO_3]$, it is obvious that true physiological conditions cannot be established in salt solutions without this buffer system. It has to be realized, however, that where the bicarbonate buffer system is in operation there are present in the solution, in certain concentrations and in certain ratios, various ions and molecules, in particular H^+, HCO_3^- and CO_3^{--} ions and CO_2 and H_2CO_3 molecules. Each of these constituents has, or may have, a specific action on living cells. Thus the cH is not the only thing that matters. To establish the

cH without the bicarbonate buffers means, therefore, that actions of other physiologically important constituents, ionic or molecular, of this system, are excluded. That such specific actions, independently of cH, do exist is certain.

There is, for example, strong experimental evidence that bicarbonate ions exert a specific stimulating effect on the rhythmic activity of the intestine (Rona and Neukirch, 1912; Sollmann *et al.*, 1928b; M'Callum and Magee, 1930). Further, on account of some findings, an action of HCO_3^- ions on the respiratory centre has been considered possible (Laqueur and Verzar, 1909; Hooker *et al.*, 1917).

Some facts have also been presented which lend support to the assumption that CO_3^{--} ions, at least in certain structures (plain muscle of the uterus), have effects which are independent of pH, HCO_3^- ion concentration or CO_2 tension (Van Dyke and Hastings, 1927–28).

Due consideration must particularly be given to possible specific actions of free CO_2 as such. Physically absorbed CO_2 in aqueous solutions is not, as is often thought, entirely hydrated; in fact, the greater proportion of it remains anhydrous, only about 1/1,000 being converted to H_2CO_3 (Buytendyk *et al.*, 1927; Van Slyke *et al.*, 1928; Stadie and O'Brian, 1937; Schmidt, 1941). Free CO_2 molecules, thus present in body fluids or artificial media, could exert specific actions, i.e. may act in other ways than by increasing the acidity of the environment. In respect of the respiratory centre, once presumed to be affected only by pH changes caused by CO_2, certain experimental evidence in favour of a direct CO_2 action has been forthcoming (Scott, 1918; Nielsen, 1936; Krogh, 1940; Schmidt, 1941; adverse criticism by others, e.g. Douglas and Havard, 1932). Direct CO_2 actions on other structures are also likely to exist. Observations by Jacobs (1919), for example, prove that CO_2 molecules *per se* have a toxic effect on protozoa and tadpoles. Some experimental findings also indicate that CO_2 acts, independently of pH, on the vessels of the yolk sac of the chick embryo (Hammet and Zoll, 1928).

There is no doubt that important CO_2 effects are due to increase in the acidity, such as the depressing action of CO_2 in higher concentrations on the automatic activity of the intestine (Fraser, 1925; Polansky, 1927; Sollmann *et al.*, 1928), but CO_2 in itself at lower concentration may probably serve as a stimulus (Mansfeld, 1921). Similarly, although the heart rate is certainly controlled by cH (Gremels and Starling, 1921; Bainbridge, 1923; Barlow, 1930) there are, on the other hand, facts which seem also to indicate some specific influence on the heart rate of free CO_2 and HCO_3^- ions (Gross, 1903; Rona and Neukirch, 1912; Mansfeld and v. Szent Györgyi, 1920; Andrews, 1924).

With regard to the findings mentioned, specific CO_2 actions on living tissues—even if partly due to the great diffusibility of CO_2—cannot be ruled out. Consequently, CO_2 has to be regarded as an essential environmental factor with possible actions beyond its rôle in the bicarbonate buffer system. Thus, CO_2 is required in physiological salt solutions not only to effect the pH regulation, but also to secure any such specific action. To make such specific effects physiological, CO_2 should be present at tensions equal to the average CO_2 tension in blood (40 to 45 mm. Hg.). That only gas mixtures containing 5 to 6 volumes p.c. CO_2 meet this requirement, has been stated above.

In the foregoing, the opinion is held that the bicarbonate buffer system is of physiological importance not only by virtue of cH it provides, but with all its constituents, in particular free CO_2. This buffer system has, therefore, to be

regarded as an environmental complex which cannot adequately be replaced by other buffer systems.

The above experiments dealing with the relationship of the pH of Tyrode's solution and the bicarbonate system have shown that in conditions of ordinary experiments, with a reasonable degree of care, particularly in providing a certain CO_2 concentration, but without any extraordinary precautions, the cH can satisfactorily be kept constant (i.e. within ± 25 p.c.) for an unlimited time and be varied within the physiological range. It appears that this form of controlling the pH of salt solutions is the only physiological method of pH control, provided the principles outlined above are observed, ensuring correct environmental conditions apart from cH.

It may be pointed out that the experiments in question refer to relatively smaller quantities of fluids and do not deal with possible pH changes caused by acid metabolites of organs. This paper is not concerned either with the question as to the optimal ionic and molecular equilibrium of salt solutions containing 5 to 6 volumes p.c. CO_2; this equilibrium will differ somewhat according to the particular structure or the particular function to be investigated.

SUMMARY.

The spontaneous changes of the reaction of Tyrode's and Fleisch's solutions were studied for long periods of time. Tyrode's solution was found to be very unstable, particularly when oxygenated, its cH falling within some hours to less than one tenth of the initial value. Fleisch's solution, although relatively stable, does not, however, meet physiological requirements for long-lasting experiments.

By varying the CO_2 supply and the bicarbonate content the pH of Tyrode's solution can, without taking extraordinary measures, be altered and kept constant with a high degree of accuracy, i.e. within 0·1 of the pH.

Reasons are stated for attributing to the bicarbonate buffer system physiological significance beyond its rôle in regulating cH.

Acknowledgment. We wish to express our thanks to Professor J. G. Wood for making available a Cambridge pH meter, with which the earlier part of this work was done.

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FACTORS AFFECTING THE DURATION OF NEMBUTAL ANAESTHESIA IN RATS

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At the end of 1946, one of us (F.H.S.) carried out a series of experiments in which were investigated various aspects of Nembutal anaesthesia in rats at different body-temperatures. A year later these experiments were repeated (by K.H.S.) in full and the results were statistically treated. Of the earlier series of tests, it is sufficient to say here that the findings were fully supported and borne out in the later series, the results of which are here presented.

In 1947, Fuhrman published a paper covering part of the field of the present investigation, and used body-temperature as the measure of thermal effects, rather than the environmental temperature as used by earlier workers. At high and low environmental temperatures, even without anaesthesia, small animals possess a fluctuating body-temperature; when they are anaesthetized, only the body temperature is a satisfactory thermal index.

Here an attempt is made to point out the effects of body temperature and sex on the anaesthetic time and the mortality in rats injected with Nembutal (sodium pentobarbital).

THE EFFECT OF BODY-TEMPERATURE.

Brunton (1874) noticed that warmth prevented death in guinea-pigs given toxic doses of chloral hydrate, and suggested treating acute chloral poisoning with warmth. He used rectal temperatures in his work but most later workers, using barbiturates, did not. Raventós (1938), using Evipal in mice, found the duration of anaesthesia was 2·5 times as long at 20° C. as at 30° C., and shorter at 40° C. than at 30° C. Cameron (1939), giving pentobarbital to rats, found that the anaesthetic-time increased as the environmental temperature decreased; he also found that warmth prevented death. Gaylord and Hodge (1944) showed a similar increase in anaesthetic-time with decrease of environmental temperature, when pentobarbital was injected subcutaneously into female rats.

[1] The preliminary portion of this work (F.H.S.) was carried out with the aid of a grant from the National Health and Medical Research Council. The investigation was continued (K.H.S.) with funds supplied by Nicholas Pty. Ltd., and Burroughs Wellcome & Co. (Australia) Ltd. We wish to thank Dr. Lancaster for checking the statistical analyses.

Fuhrman (1946) suggested that the difference in sleeping times at higher and lower temperatures may be due to a decreased rate of detoxification of the barbiturate at lower temperatures. With pentobarbital he found the mean sleeping time at 37° C. to be 17·5 minutes, while at 27° C. it was 61·8 minutes, significantly different values. He used 3·75 mg./100 gm. body-weight of sodium pentobarbiturate injected into white mice intraperitoneally, and used body-temperatures, which were maintained constant by special means. However, all these results were obtained at one dose level. It is our intention to investigate a range of dosages. That this is necessary is very evident from our results.

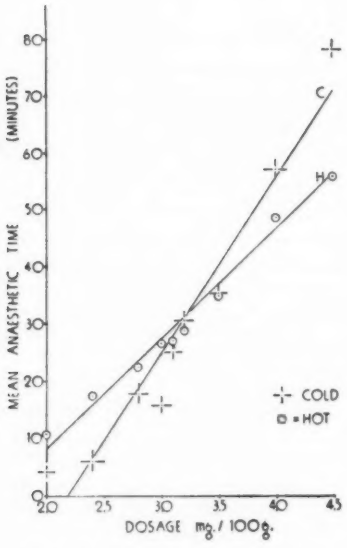

Fig. 1. Dosage: Anaesthetic time curve at two levels of body-temperature. Regression lines for results at the two levels are shown.

Experimental.

A group of adult white rats, consisting of 40 males at the dosage levels of 2·4 and 2·8 mg./100 gm. body-weight and at other dosage levels of 32 males and 8 females, was used. The sexes were kept separate. Taken eight at a time, in two cages of four for convenience in handling, they were put into a refrigerator, the internal temperature of which was 7° C., and left for 30 minutes. Taken four at a time, each was rapidly weighed and injected intra-peritoneally with a solution of Nembutal in 0·9 p.c. sodium chloride solution, the dose being proportionate to the body-weight. The Nembutal solutions were so made that the dose for a rat of 250 gm. body-weight was contained in 0·5 ml. solution. As soon as they were injected, the rats were marked for identification and returned to the refrigerator for a further 15 minutes. The time of injection was noted, together with the time of induction of anaesthesia and the rectal temperature of any rats becoming anaesthetic; should the rectal temperature fall below 32° C. the rat was wrapped in cotton wool and placed on the bench at room temperature until the rectal temperature was above 32° C., when it was replaced in the refrigerator. In any case, the rats were removed, 15 minutes after injection, to the bench, their rectal temperatures were noted, and they were allowed to recover—once more being wrapped in cotton wool should their body-temperatures fall below 32° C. The total time of anaesthesia (taken from the time of induction to the return of the righting reflex) was recorded for each rat. Every few minutes the rats were turned over, and, when they showed signs of returning consciousness, were stimulated gently by bending their tails; this was done to prevent them passing into a post-anaesthetic sleep without visible sign of the end of anaesthesia.

When all 40 rats had been treated in this way, the experiment was repeated with the same dosage of Nembutal, the rats being put for 20 minutes into an incubator at 38·5–40° C., weighed, injected and marked, and returned to the incubator for 15 minutes. The procedure was then as above. Oxygen was trickled into the incubator while the rats were being treated; it was found that this prevented deaths during the pre-injection heating (which caused a rise in the body-temperature).

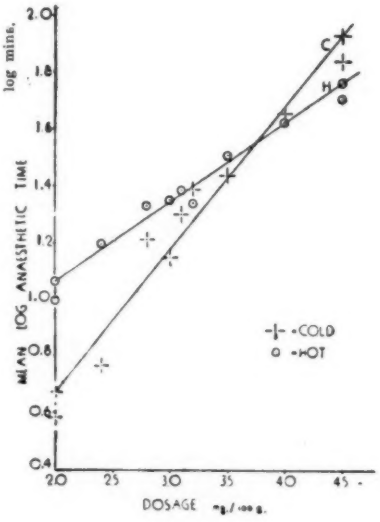

Fig. 2. Dosage: Mean log anaesthetic time curve at two levels of body-temperature. Showing regression lines for results at each level.

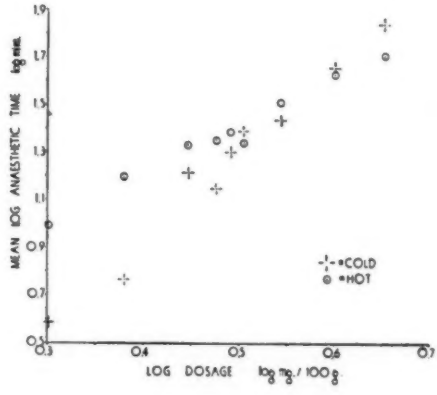

Fig. 3. Log-dosage: Mean log anaesthetic time graph, showing crossing trends of points for each of two levels of body-temperature.

The experiment was then repeated in both refrigerator and incubator for each of a series of dosages. For dosages of 2·0–3·0 mg. Nembutal/100 gm., the rats were used on consecutive days; for dosages in excess of 3·0 mg./100 gm., at least one clear day was allowed between treatments.

It should be noted that throughout this paper by "dosage" is meant "so many mg./100 gm. body-weight" and by "dose" is meant the actual amount given in mg. A rat of 150 gm. would be given a dose of 4·5 mg. at a dosage of 3·0 mg./100 gm. body-weight. Also, dosage is expressed throughout as mg. Nembutal/100 gm., not as mg. barbituric acid.

The results of this section of the experiment are given in Table 1, while Figs. 1 to 3 show relationships between the dosage and the anaesthetic time at two levels of mean body-temperatures, 33·58° C. (cold) and 39·77° C. (hot).

TABLE 1.

Dosage mg./100 gm.	Log dosage.	Anaesthesia induced out of 40		p.c.		Mean anaesthetic time, minutes.		Mean log anaesthetic time, minutes.	
		Cold.	Hot.	Cold.	Hot.	Cold.	Hot.	Cold.	Hot.
2·0	·3010	8	25	20	62·5	4·125	10·68	0·5875	0·9942
2·4	·3802	14	29	35	72·5	6·0	17·3	0·7658	1·1989
2·8	·4472	34	32	85	80	17·7	22·4	1·2154	1·3314
3·0	·4771	37	30	92·5	75	15·78	26·6	1·1493	1·3537
3·1	·4914	37	39	92·5	97·5	25·1	27·0	1·3018	1·3881
3·2	·5051	38	30	95	75	30·5	28·9	1·3896	1·3408
3·5	·5441	40	40	100	100	35·2	34·8	1·4394	1·5117
4·0	·6021	39	39	97·5	97·5	57·1	48·5	1·6592	1·6291
4·5	·6532	40	40	100	100	78·3	55·9	1·8399	1·7079

Fig. 1 shows mean anaesthetic-times plotted against the dosage; however, as the anaesthetic-times at each dosage level were not normally distributed but showed a skewness, the means of the logarithms of the anaesthetic times were calculated and are given in Table 1. Figs. 2 and 3 are of curves involving these log-anaesthetic-times and the dosage and the log-dosage respectively.

Test of Significance of Difference between Regression Coefficients.

Of Fig. 1:

COLD

$\Sigma X = 28{\cdot}5$

$\Sigma Y_c = 269{\cdot}805$

$\Sigma XY_c = 998{\cdot}91$

$\Sigma xy_c = 144{\cdot}54$

$\Sigma X^2 = 94{\cdot}95$

$\Sigma x^2 = 4{\cdot}71$

$\Sigma Y_c^2 = 12{,}805{\cdot}61$

$\Sigma y_c^2 = 4{,}737{\cdot}20$

Equn.: $Y_c^1 = 30{\cdot}75X - 67{\cdot}38$

$\Sigma(Y_c - Y_c^1) = 272{\cdot}06$

$s_{v_c}^2 = \frac{272{\cdot}06}{8} = 34{\cdot}01$

HOT

$\Sigma X = 28{\cdot}5$

$\Sigma Y_H = 272{\cdot}08$

$\Sigma XY_H = 950{\cdot}93$

$\Sigma xy_H = 89{\cdot}37$

$\Sigma X^2 = 94{\cdot}95$

$\Sigma x^2 = 4{\cdot}71$

$\Sigma Y_H^2 = 9{,}875{\cdot}0$

$\Sigma y_H^2 = 1{,}650{\cdot}02$

Equn.: $Y_H^1 = 19{\cdot}01X - 29{\cdot}96$

$\Sigma(Y_H - Y_H^1) = 28{\cdot}157$

$s_{v_H}^2 = \frac{28{\cdot}157}{8} = 3{\cdot}52$

$$\therefore \sigma_{b_c - b_H} = \sqrt{\frac{34{\cdot}01}{4{\cdot}71} + \frac{3{\cdot}52}{4{\cdot}71}} = 2{\cdot}823$$

$$t = \frac{b_c - b_H}{\sigma_{b_c - b_H}} = 4{\cdot}16$$

From Tables, t ($p = 0{\cdot}05$, $d.f. = 7$) is 2·365
and t ($p = 0{\cdot}01$, $d.f. = 7$) is 3·499

There is then a highly significant difference between the regression coefficients of the curves in Fig. 1.

Of Fig. 2:

COLD		HOT	
ΣX	$= 28 \cdot 5$	ΣX	$= 28 \cdot 5$
ΣY_c	$= 11 \cdot 3479$	ΣY_H	$= 12 \cdot 4558$
ΣXY_c	$= 38 \cdot 30049$	ΣXY_H	$= 40 \cdot 74135$
Σxy_c	$= 0 \cdot 96765$	Σxy_H	$= 1 \cdot 25646$
ΣX^2	$= 94 \cdot 95$	ΣX^2	$= 94 \cdot 95$
Σx^2	$= 4 \cdot 71$	Σx^2	$= 4 \cdot 71$
ΣY_c^2	$= 15 \cdot 4606$	ΣY_H^2	$= 17 \cdot 4055$
Σy_c^2	$= 1 \cdot 1520$	Σy_H^2	$= 0 \cdot 1667$
Equn.: Y_c^1	$= 0 \cdot 5033X - 0 \cdot 3329$	Equn.: Y_H^1	$= 0 \cdot 2762X + 0 \cdot 5095$
$\Sigma(Y_c - Y_c^1)$	$= 0 \cdot 66626$	$\Sigma(Y_H - Y_H^1)$	$= 0 \cdot 015572$
$S^2_{y_c}$	$= 0 \cdot 0083285$	$S^2_{y_H}$	$= 0 \cdot 0019465$

$$\sigma_{b_c - b_H} = \sqrt{\frac{0 \cdot 0102750}{4 \cdot 71}} = \sqrt{0 \cdot 002181} = 0 \cdot 0467$$

$$t = \frac{0 \cdot 2271}{0 \cdot 0467} = 4 \cdot 863$$

From Tables, t ($p = 0 \cdot 01$, $d.f. = 7$) is $3 \cdot 499$.

There is, therefore, a highly significant difference between the regression coefficients of the curves in Fig. 2.

The results indicate a significant difference between the slopes of the regression lines of dosage response and log dosage-response curves for Nembutal anaesthesia at different levels of body-temperature. We can infer from the curves then that low dosages of Nembutal are more effective at high body-temperatures and high dosages at low body-temperatures. It is suggested then that there are two ways in which the duration of Nembutal anaesthesia may be controlled in practice; the first is by maintaining the body-temperature at a given level while varying the dosage, and the second entails the use of a given dosage while varying the body-temperature (e.g., by pre-anaesthetic conditioning and/or post-anaesthetic control of environmental temperature).

Another point of great interest is the fact that at the low dose levels induction is less frequent in the cold than at higher temperatures. Table 1 (columns 5 and 6) shows a very significant difference (chi square test) between the percentage actually anaesthetized in the cold and hot environments at the dose levels 2·0–2·4 mg./100 gm.

VARIATION DUE TO SEX AND TO WEIGHT (IN MALES).

Fitch and Tatum (1932) concluded that there was no difference in anaesthetic-time due to sex. Holck and Kanân (1935) found female white rats were more sensitive to Nembutal than were males, and on any given dosage were

more apt to die and slept longer. Moir (1937) said that the average sleeping time of females was greater than that of males; though very young females were more resistant than very young males, the resistance disappeared with age, mature females being less resistant than were mature males. Carmichael (1938) found no difference due to sex in the LD_{50} for either young or old rats. Homburger, Etsen and Himwich (1947) noted that as growth proceeded the females showed an increase in susceptibility to pentobarbiturate in the weight range of 101–200 gm., the females remaining in light anaesthesia longer than males, though the lethality was the same for each sex; in the weight range of 201–500 gm. the females showed both a longer duration of narcosis and a greater lethality than did males.

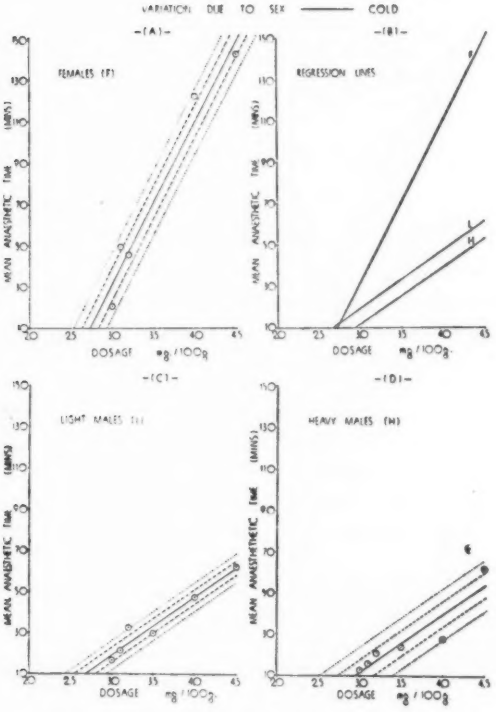

Fig. 4. Variation in anaesthetic-time due to Sex: Cold.

A. Females ——— Regression line.
C. Light Males ------ ± 1 Standard Error.
D. Heavy Males ..... ± 2 Standard Error.
B. Superimposed regression lines of A, C, and D.

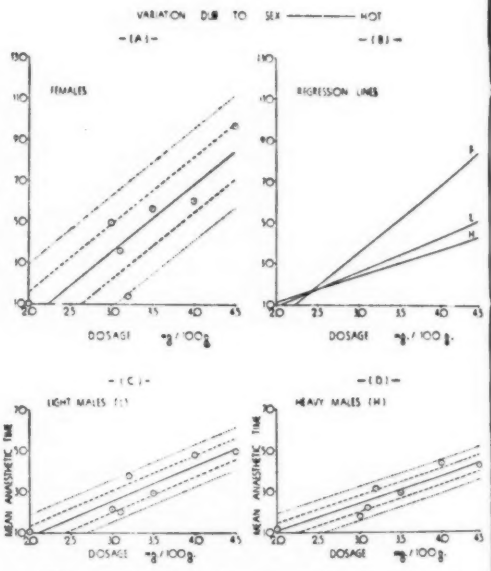

Fig. 5. Variation in anaesthetic time due to Sex: Hot.

A. Females ——— Regression line.
C. Light Males ------ ± 1 Standard Error.
D. Heavy Males ..... ± 2 Standard Error.
B. Superimposed regression lines of A, C, and D.

Experimental.

The results obtained in the manner described earlier for dosages of 2·0–4·5 mg./100 gm. body-weight in the cold and the hot conditions of body-temperature, and at 2·0 mg./100 gm. in the hot only were used for this investigation.

The female rats used, though mature, were of lower body-weight than were the males, on the whole; there was some overlap but not sufficient to obviate the possibility of weight-induced variation on the anaesthetic-time. A trial graph showed a marked divergence of the curves for males and females. The males were then split into two arbitrary groups about their mean body-weight, and the results for the Light (L) and Heavy (H) groups of males were plotted with those for the Female (F) group (Figs. 4 and 5). Though the body-weights in the L and F groups overlapped, the curves show the regression lines for the two male groups close together and well removed from that of the females, so that it would seem that there was little weight-induced variation but quite an appreciable variation due to sex.

TABLE 2.

Sex variation and male weight variation.

Dosage mg./100 gm.	Mean anaesthetic time (minutes). Cold. 33·65° C.) F.	L.	H.	Hot. 39·32° C.) F.	L.	H.
2·0	—	—	—	10·125	10·38	11·5
3·0	20·875	16·38	12·38	49·57	21·43	17·69
3·1	49·5	21·155	15·675	35·75	21·09	21·93
3·2	45·5	32·44	20·69	13·875	37·53	31·10
3·5	69·125	29·70	23·67	56·375	29·26	28·98
4·0	120·25	47·48	27·61	60·125	47·89	43·21
4·5	142·75	62·34	62·03	96·875	49·34	41·90
Totals	448·000	209·495	162·055	322·695	216·92	196·31
Means	74·66	34·916	27·009	46·099	30·99	28·04
Mean dosage (X)		3·55			3·33	

From these were calculated regression lines, with their Standard Errors, and the significance of difference of their slopes (b's).

Cold: For F: $Y = 78·72X - 204·80$ $\quad S_Y = 7·83$
For L: $Y = 28·37X - 65·80$ $\quad S_Y = 3·59$
For H: $Y = 28·28X - 73·38$ $\quad S_Y = 6·23$
Using F and L only, $\sigma_{b_F - b_L} = 2·566$
$t = 19·6$
From Tables, ($p = 0·001$, $n = 10$), $t = 4·587$

Hot: For F: $Y = 32·63X - 62·512$ $\quad S_Y = 13·57$
For L: $Y = 16·99X - 25·564$ $\quad S_Y = 5·15$
For H: $Y = 12·90X - 14·89$ $\quad S_Y = 4·06$
Using F and L only, $\sigma_{b_F - b_L} = 2·231$
$t = 7·01$
Using F and H only, $\sigma_{b_F - b_H} = 2·163$
$t = 9·11$
Using L and H only, $\sigma_{b_L - b_H} = 2·449$
$t = 1·670$
From Tables, ($p = 0·05$, $n = 12$), $t = 2·18$

In each case where the slopes of the regression lines for the female and a male group have been compared, there has been shown a high degree of significance of difference, indicating that there was a difference, unlikely to be due to chance (odds of less than 5 in 10,000), in the extent to which the sexes respond to Nembutal.

When the two groups of males were similarly compared, no significant difference was found. This may show that the difference shown between the slopes of the regression lines of the females and males is due mainly to sex and not to weight.

The difference between the slopes, when the two sexes are considered, is more marked at the lower body-temperature than at the higher, as may be seen by a direct comparison of the values of b, as well as by the comparison of the calculated t-values.

EFFECT OF BODY-TEMPERATURE ON MORTALITY.

In the earlier series of experiments dealt with here, F.H.S. obtained figures for the mortality rates at different levels of body-temperature when a given dosage of Nembutal was used with rats. His results are shown in Table 3, and graphically in Fig. 6.

TABLE 3.

The effect of body-temperature on mortality.

Body temperature. °C.	Number killed.	p.c. killed.
32	12/25	48
35	9/28	32
38·5	5/24	21
39·5	16/30	53
40·5	9/9	100

Dosage used: 7 mg./100 gm.

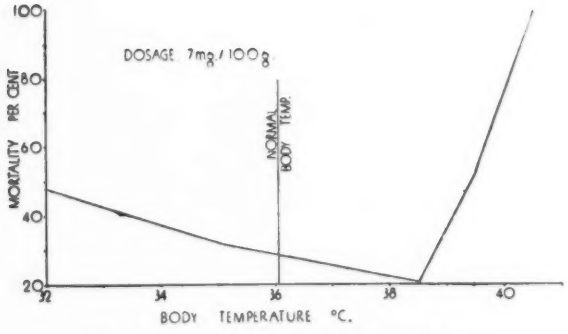

Fig. 6. Body-temperature: Mortality curve for a single dosage.

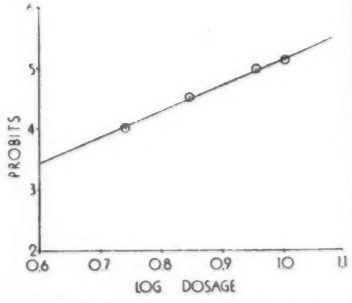

Fig. 7. Log dosage: Probits curve. Line fitted by eye to plotted points.

From the graph, it is seen that as the body-temperature is raised there is a decrease in the observed mortality up to a point which lies at a body-temperature of between 38° C. and 39° C. Above this there is a marked increase in the mortality rate for even a small increase in body-temperature; this is probably not entirely due to alteration in the efficacy of the Nembutal at the raised temperature, but to a large extent to the body-temperature being raised to a level at which rats begin dying from conditions due to hyperthermia. In this range of body-temperatures cooling measures will decrease the mortality; at lower temperatures, warmth should be applied.

In sum, for a dosage of 7·0 mg./100 gm. body-weight, in rats, the body-temperature should be brought to 37·5–39·0° C. if one wishes to keep the mortality rate as low as possible.

DETERMINATION OF AN LD_{50}.

Barlow, Duncan and Gledhill (1931) found the minimum lethal dose for sodium pentobarbiturate to be 12·0 mg./100 gm. on subcutaneous injection; Swanson and Shonle (1931) reported the same value for intraperitoneal injection, while Fitch and Tatum (1932) found that intraperitoneal injection of 7·5 mg./100 gm. gave a 50 p.c. kill. Carmichael (1938) determined the LD_{50} for young rats as 11·0–12·0 mg./100 gm. and for old rats as 8·5–9·5 mg./100 gm.

From the results of the earlier set of experiments (F.H.S.), a value for the LD_{50} is obtained. The results are given in the first four columns of Table 4, and from them, by the method of Bliss (1938) is calculated the LD_{50}. Fig. 7 shows the empirical probits equivalent to the percentage mortalities plotted against the log-dosages; the eye-fitted line gives the equation

$$Y_B = 4{\cdot}28x + 0{\cdot}842$$

from which are calculated the expected probits.

The numbered equations are given the numbers of the corresponding theoretical equations in Bliss's paper.

TABLE 4.

Dosage mg./100 gm. X.	Log dosage x.	Mortality	p.c. M	Empirical probit.	Expected probit. Y.	Working probit. y.	Weight w.
5·5	0·7404	3/32	16	4·0055	4·010	4·006	14·04
7·0	0·8451	9/28	32	4·5323	4·459	4·533	16·27
9·0	0·9542	10/20	50	5·0000	4·925	5·000	12·69
11·0	1·0414	22/41	55	5·1257	5·296	5·122	25·26
					Total	(rounded)	68·25

$$\bar{x} = \frac{Swx}{Sw} = 0{\cdot}9164 \quad \text{Equn. 4} \qquad \bar{y} = \frac{Swy}{Sw} = 4{\cdot}508 \quad \text{Equn. 5}$$

$$[wx^2] = S(wx^2) - \bar{x}S(wx) = 0{\cdot}90 \quad \text{Equn. 6.}$$

$$[wxy] = S(wxy) - \bar{y}S(wx) = 17{\cdot}3 \quad \text{Equn. 7.}$$

$$s = \frac{[wx^2]}{[wxy]} = \frac{1}{b} = 0{\cdot}05202 \quad \text{Equn. 16.}$$

$$\log \text{LD}_{50} = 0{\cdot}9164 + 0{\cdot}05202\ (5 - 4{\cdot}508) = 0{\cdot}9420. \quad \text{Equn. 17.}$$

$$\text{LD}_{50} = 8{\cdot}75 \text{ mg./100 gm.}$$

Chi-square test: $\chi^2 = [wy^2] - b[wxy]$ Equn. 11.

$$= 1471{\cdot}1 - \frac{17{\cdot}3}{0{\cdot}9}\,17{\cdot}3$$

$$= 1471{\cdot}1 - 332{\cdot}5$$

$$= 1138{\cdot}6$$

From Tables, for $n = 2$, $p = 0{\cdot}001$, $\chi^2 = 13{\cdot}8$.

Standard error of $\log \text{LD}_{50}$:

$$V_{\log \text{LD}_{50}} = s^2\left\{\frac{s(5-\bar{y})^2}{wxy} + \frac{1}{S(w)}\right\}. \quad \text{Equn. 20.}$$

$$= 0{\cdot}052022\left\{0{\cdot}05202\,\frac{0{\cdot}492}{17{\cdot}3} + \frac{1}{68{\cdot}25}\right\}$$

$$= 0{\cdot}00004162$$

$$SE_{\log \text{LD}_{50}} = 0{\cdot}006452$$

Then, for a range of $\pm 3SE_{\log \text{LD}_{50}}$, the limits of the $\log \text{LD}_{50}$ are 0·9615 and 0·9225.

Therefore the effective range of the LD_{50} is from 8·37 to 9·15 mg./100 gm., with the average value at 8·75 mg./100 gm.

The determined LD_{50}, 8·75 mg./100 gm. (with range of 8·37 to 9·15 mg./100gm.), is intermediate among the previously published results; in particular it agrees very well with the value obtained by Carmichael.

SUMMARY.

There is a significant difference between the slopes of the regression lines of dosage-response and log dosage-response curves for Nembutal anaesthesia at different levels of body-temperature.

It is suggested that this could be made use of in controlling the duration of anaesthesia.

A significant difference in the response of the sexes is noted. This difference is more marked at low body-temperatures than at higher.

No variation due to weight in the response was seen; but it must be remembered that the experiment was not planned to assess such variation.

For a given dosage of Nembutal, the mortality rate varies over a range of temperatures. For a dosage of 7·0 mg./100 gm. body-weight, the lowest mortality is given at a temperature of 37·5 to 39·0° C.

It is suggested that this variation of mortality rate over a temperature-range should be used to control mortality due to Nembutal.

The LD_{50} is determined as lying within the range 8·37 to 9·15 mg./100 gm. body-weight, the average value found being 8·75 mg./100 gm.

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SODIUM, POTASSIUM BALANCE IN CELLS

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Recent research has indicated that the characteristic distribution of sodium and potassium in animal cells may be regarded as a result of the physico-chemical balance between the protoplasm and the medium surrounding the cell rather than a specific membrane effect (Steinbach, 1940). It is becoming increasingly evident that cell membranes are considerably more permeable to ions of various species than was thought previously (Steinbach, 1940; Brooks, 1945).

It is known that proteins bind cations and that the affinity of the protein for different cations varies, and it was thought that the distribution may be brought about, in some measure, by the protein present in the cell. This idea is not new, for in 1912 Moore, Roaf and Webster wrote, "The varying concentrations of sodium, potassium and phosphatic ions within and without the cell are an expression of specific affinities of the definite colloids of each particular cell type for these ions, and do not mean that there is a membrane acting as a gate to these ions." As far as can be ascertained from the literature, differences in the affinities of cellular proteins for sodium as opposed to potassium have not been observed under satisfactory conditions.

In an attempt to test the hypothesis *in vitro*, experiments were carried out in which a protein solution, in this case haemoglobin, was separated by a semi-permeable membrane from an ionic solution such as Ringer's fluid.

METHOD.

The apparatus consisted essentially of a small inside volume (approx. 25-30 ml.) in which was contained the protein solution separated by a semi-permeable membrane from a large outside volume of the ionic solution. Large volumes were used to ensure constant ionic concentrations in the outside solution; both solutions were kept thoroughly mixed by mechanical stirring. The haemoglobin used in the experiments was obtained by haemolysing washed red-cells (equine) with ether and, after removal of the ether, dialysing for a period of 4–5 days against running water to remove diffusible cations originally present. During the dialysis procedure it was observed that while sodium dialysed out quickly and little or none remained after 3–4 days, potassium, on the other hand, dialysed out more slowly and quite appreciable quantities remained even after dialysing for periods of up to eight days. This, in itself, indicates a stronger affinity of haemoglobin for potassium than for sodium.

In the experiments concentrations of haemoglobin ranging from 5–20 p.c. (estimated

[1] Technical assistance was provided by a grant from the National Health and Medical Research Council.

colorimetrically by the acid haematin method) were used. Ringer's solution was employed as the ionic solution, the composition of which was: NaCl 7 gm., KCl 0·14 gm., $NaHCO_3$ 0·2 gm., water to 1,000 ml. The membranes used were parchment or cellophane of such permeability that if distilled water was placed in the inside cell normally containing the protein solution, equilibrium was reached within 2–3 hours.

Two series of experiments were carried out. In the first the experiment was allowed to continue until equilibrium had been reached, usually in approximately eight hours at 25° C., and the protein solution analysed. In the second series larger volumes of haemoglobin solution were used (up to 100 ml.) and samples were taken and analysed at intervals throughout the course of the experiment. The pH of both solutions in each series of experiments was the same, viz. 7·2. As the second series of experiments were run for long periods—up to 14 days—they were carried out at low temperatures to avoid microbial growth. Sodium was determined by the standard zinc uranyl acetate method (Peters and Van Slyke, 1932), while potassium was determined by a modification of the cobalti-nitrite method (to be published later).

RESULTS.

The results of both series of experiments show that potassium will accumulate in the inside cell against a concentration gradient and in the presence of relatively large quantities of sodium (Table 1). The greatest accumulation observed was to an extent of three times the concentration present in the external solution, that is, the concentration of potassium inside, at the end of the experiment, was 20 mg./100 ml., while that outside was only 7 mg./100 ml. In no case did the concentration of sodium inside the cell reach that outside; which, of course, would be expected from membrane equilibria considerations.

TABLE 1.

Showing distribution at equilibrium of potassium and sodium in the inside cell at various haemoglobin concentrations, and at different temperatures. In all cases the initial concentrations of potassium present in the haemoglobin solutions were less than 6 mg./100 ml.

The concentrations of sodium and potassium in the Ringer's solution were 7 mg./100 ml. of potassium and 280 mg./100 ml. sodium.

Expt. No.	Haemoglobin conc. p.c.	Temperature ° C.	Type of membrane	Potassium conc. inside (mg./100 ml.)	Sodium conc. inside (mg./100 ml.)
1	6	30	Parchment	8	200
2	6	25	Parchment	8	193
3	8	26	Cellophane	11	154
4	9·5	30	Cellophane	14·5	122
5	2	25	Cellophane	6·6	234
6	9·5	35	Parchment	13	155
7	12	25	Parchment	16·3	119·1
8	14	20	Parchment	13	154
9	14	30	Parchment	15	187
10	18	25	Cellophane	17·5	106
11	19	15	Parchment	15·5	160
12	20	15	Parchment	20	107
13	9	2	Cellophane	14	184

Equilibrium appeared to be reached in approximately 8 hours and the high concentration of potassium in the protein was maintained for periods of at least 24 hours after which time it commenced to fall off gradually, falling to approximately the outside level. In two cases, however, the high concentration of potassium remained for three and a half days. It

was found that the concentration of 20 mg./100 ml. was observed in 20 p.c. haemoglobin solution while with lower concentrations the accumulation in most cases tended to be proportionally lower, e.g. in 14 p.c. haemoglobin the concentration of potassium was 15 mg./100 ml. There were no appreciable differences in equilibrium conditions between experiments carried out at different temperatures between 2° C. and 37° C.

DISCUSSION.

The concentration of potassium in equine red blood corpuscles is thirteen times that in the plasma, and sodium is the reverse (Table 2). This cannot be accounted for by membrane permeability, unless the membrane is impermeable to sodium but is permeable to potassium. The behaviour of ions in corpuscles in potassium deficiency, abnormal salt solutions and to isotopes indicates that this is not the case. The results of the present experiments indicate that equine haemoglobin has a higher affinity for potassium than for sodium. The degree of differential association is not of the magnitude found in cells, but the concentration of haemoglobin was only 60 p.c. of that present in cells. It is impossible to infer the behaviour of haemoglobin at 30 p.c. concentration from our observations. They do, however, raise the possibility that intracellular concentrations of potassium are due, at least partly, to differential association of potassium with the native proteins.

TABLE 2.

Showing the distribution of sodium and potassium between equine erythrocytes and serum calculated from the results of Abderhalden (1898). It will be noted that the distribution is similar to that in human blood.

	gm./1,000 gm. serum	gm./1,000 gm. cells
Sodium	3·23	—
Potassium	0·211	2·76

SUMMARY.

Haemoglobin solutions were dialysed against Ringer's solution and potassium was observed to accumulate in the haemoglobin solutions against a concentration gradient. Comparison is made between the experimental observations and conditions obtaining in the living cell.

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CARDIAC ANEURYSMS

A MECHANICAL ANALYSIS OF THEIR FORMATION

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(Accepted for publication, 30th June, 1948.)

For many years aneurysm formation in the walls of the ventricles of the heart has been recognized as a sequel of myocardial infarction. The consequences and clinical effects of such deformation of the ventricular wall have been reviewed by Legg (1883), Parkinson, Bedford and Thomson (1938), and Diaz (1946). Little consideration, however, has been given to the exact mode of development of the deformity in the wall, or to the types of infarction which lead to aneurysm formation.

In 1941 one of us (T.E.L.) concluded from an empirical study of laminar scars in the myocardium of several hearts, that for a basal aneurysm to develop in the left ventricle it was necessary to have destruction of two of the deep muscle layers whereas at the apex of the ventricle involvement of only one muscle was necessary. These findings were considered to be in accord with the laminated arrangement of the ventricular muscles. It was also concluded that thinning of the ventricular wall without aneurysm formation resulted from involvement of one deep muscle alone.

The detailed arrangements of the muscles in the ventricular walls are now definitely established (Mall, 1910; Flett, 1927; Robb, 1934; Lowe, 1939) and much is known about the physiological behaviour of cardiac muscle (Lundin, 1944). The distortion in the ventricular wall after infarction is considered to be purely mechanical, i.e. such as to satisfy the requirements of equilibrium of the mechanical forces involved. In this paper an analysis of the distortions expected to occur after infarcts of various types and sizes is presented. These calculated distortions are compared with those occurring in the natural course of the disease and some general principles concerning the development of cardiac aneurysms are elaborated.

THEORETICAL CONSIDERATIONS.

Structure of the Ventricular Wall.

The ventricular muscle mass is composed of a series of muscles arranged in layers and running in a spiral fashion around the ventricular cavities. All these muscles arise from and are inserted in the auriculo-ventricular ring or its continuations. Although various workers

give minor differences in the descriptions of these muscles they may be conveniently grouped into four—the Superficial and Deep, Bulbo- and Sino-spiral muscles.[1] Of these, the Deep Bulbospiral muscle is confined to the wall of the left ventricle but the others are common to both ventricles.

The direction of the fibres in the muscles varies from place to place within each muscle and in the different muscles but, for the present analysis, they may be considered to form a laminated four-layer wall for the left ventricle and a three-layer one for the right. The thickness of these muscles in the average normal adult heart has been determined by Robb (1938) who finds that for the left ventricle the average thickness of the Superficial Bulbo- and Sino-spiral muscle is 1 mm., for the Deep Bulbospiral muscle 6 mm., and for the Deep Sino-spiral muscle 3 mm., giving a total thickness of 11 mm. for the ventricular wall.

The anastomosing nature of the cardiac muscle fibres makes each of these muscles into sheets of interlocking fibres which are arranged in the form of a latticework. The effect of this lattice is to give to the layer a considerable strength at right angles to the general line of the fibres. When, therefore, a segment of a layer is destroyed the normal fibres at its margin are unable to retract to any appreciable degree.

Physical Nature of a Mycocardial Infarct.

Infarction in cardiac muscle produces at first a coagulation necrosis of the involved tissue which loses its power of contraction but retains some tensile strength. Gradually this dead tissue becomes liquefied by autolysis and cellular reaction arising from the neighbouring healthy tissue. As a result of this discontinuity of living tissue proliferative changes occur in the tissue at the margin of the infarct and gradually fibroblasts and capillaries grow into the damaged area and will ultimately replace it by mature fibrous tissue. At a later stage this collagen-producing tissue dehydrates and the end result will be a dense scar of fibrous tissue.

There will be at first a progressive decrease in the tensile strength of the infarcted tissue and, after a time, a gradually increasing tensile strength in the newly-formed fibrous tissue as the collagen fibres develop so that ultimately the damaged muscle is replaced by a tissue of very considerable strength. As these processes are not simultaneous there will be a period when the strength of the damaged area is very low and at this time ventricular rupture after infarction is most common.

Histological studies (Lowe, 1941) of infarcted areas at various periods after the onset suggest that there is a period when the infarct may be considered to have the mechanical properties of a liquid, i.e. it is incompressible, transmits pressure equally in all directions, and so has neither rigidity nor tensile strength.

The physical extent of infarcts in the myocardium varies greatly from case to case but they may be grouped on the criteria that they involve single muscles, combinations of muscles or full thickness of the ventricular wall. For the present analysis only infarcts involving one or both of the deep muscles (i.e. laminar infarcts) are considered in detail and for convenience it is assumed that a circular portion of the muscle is involved. It is assumed that the volume of the "liquid" phase of the infarct is equal to that of the muscle tissue destroyed.

In considering the cycle of action of the ventricles there is one period in which for a short time the muscles and the contained blood may be considered to be static. This is during the isometric phase of contraction immediately before the aortic valves open. At this

[1] These muscles are referred to as S.S.S., S.B.S., D.B.S. and D.S.S. in the text.

moment the ventricular muscles have developed their maximum tensile stress, for, once the expulsion of blood from the ventricles commences the ventricular muscle contraction becomes isotonic, and the actual value of the tensile stress does not increase beyond the maximum isometric value. It is this moment, immediately preceding the opening of the aortic valves, which is selected for examination in this analysis. The pressure of the contained blood in the left ventricle will then be the diastolic arterial pressure.

That a phase of isotonic contraction follows that of isometric contraction is, perhaps, not self-evident for the intraventricular pressure rises during systole. However, during systole, ejection of blood into the aorta reduces the volume of the ventricular cavity and hence its radius; further the volume of the ventricular muscle is constant and the thickness of the wall must increase as the internal volume diminishes. These two changes allow the internal pressure to rise without increase of tension in the wall. See equation (4) on p. 502.

The distortions produced in the left ventricular wall following infarction of muscle have been calculated in a variety of cases. In two examples, deformations have been calculated for hearts with hypertrophic muscles at "the limit of hypertrophy" (Lowe and Bate, 1948). With these two exceptions the myocardium is assumed to be normal except for the area of infarction.

IDEALIZATION AND ASSUMPTIONS.

In order to make possible the calculation of the distortion of the left ventricle following infarction, the ventricle is supposed replaced by a relatively simple mechanical model to which it approximates at the phase considered (Patterson and Starling, 1914).

The model consists of three concentric spherical layers of flexible material, which are in tension because of pressure inside the inmost, and each layer just fits inside the next (Fig. 1). These three layers correspond to the four muscle layers of the ventricular wall in the following manner. The middle layer of the model represents the one or two muscle layers damaged by infarction, the outer layer of the model the intact muscle layer or layers outside that damaged, and the inner layer of the model the intact muscle layer or layers inside that damaged.

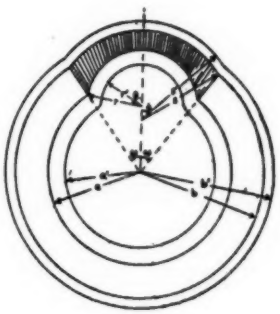

Fig. 1.

Fig. 2.

Fig. 1. Drawing of a section of the model ventricle showing the notation used in the analysis. Infarct area is shaded in all drawings.

Fig. 2. Drawing of a segment of the model wall showing the forces acting on the inner layer.

Representing an infarct in the ventricular wall, a circular cap of the middle layer of the model is supposed removed and replaced by liquid of the same volume. If the area of damage is not too great, the layers retain approximately their former shape and size (at the phase considered) except directly over and under the liquid, where the intact layers adjust to the steeper pressure gradient through them by bulging.

Equilibrium of the Ventricular Wall.

From this model it is possible to discuss the mechanical equilibrium of the forces existing in the ventricular wall. Any portion of the inner layer of this idealized ventricle, e.g. that cut off by a cone with vertex at the centre (Fig. 2), is in equilibrium. It is subject to inner and outer pressures, exerted by the blood within and the next layer without, and around its edges to tensions exerted by the remainder of the inner layer itself. Because of the curvature of this layer, these edge tensions have an inwardly directed radial resultant and permit the outer pressure to be smaller than the inner for they are antagonistic to the inner pressure.

In the same way the tension in the middle layer supports a pressure difference between the inner and outer surfaces of the layer, and the tension in the outer layer supports a pressure difference between the inner surface of this layer and its outer surface at pericardial pressure. Thus the whole ventricular wall supports the pressure of the blood within, the tension in each layer contributing part of this support.

Now suppose that portion of the middle layer shown in Figs. 1 and 2 is infarcted. This portion, being liquid, can no longer support a pressure difference, yet the same total pressure difference as before has to be supported by the whole wall. Consequently the inner and outer layers must support greater pressure differences than before.

Considering the piece of the inner layer shown in Figs. 1 and 2 the resultant of the pressures is a greater outward force than before so that a bulge develops under the infarct.

As the inner layer bulges its curvature increases, and so the directions of the edge tensions rotate inwards towards one another. This increases their inward resultant which tends to balance the increased outward force due to the greater pressure to be supported. Another factor which has this same effect is the stretching of the layer entailed by bulging. This stretching increases the edge tensions in accordance with the tension-length relation for cardiac muscle. These two factors together bring the bulging layer to equilibrium. In small bulges the former factor is the important one, as negligible stretching of the muscle occurs. In large bulges the increased tension becomes of considerable importance owing to the degree of stretching of the muscle.

Similar considerations govern the equilibrium of the outer layer. They do not, however, completely determine the equilibrium state for the whole wall, for this depends also on the volume of the liquid in the infarct region. If this volume is much greater than that of the muscle it replaces, the outer layer will bulge much more than the inner and will carry most of the total pressure difference supported by the wall.

These considerations of the conditions of equilibrium and the assumption as to the volume of the liquid in the infarct give two equations which determine the two unknowns of this problem: the height of the inner and outer bulges. The magnitude of these bulges is specified by "height factors" which are the ratio of the height of the bulge above a plane through its edge to the undistorted height above the same plane.

Some deformation in the nature of a neck surrounding the main bulge is also expected near the edge of the infarct. This "secondary" deformation is disregarded, since its effect on the main bulge is probably slight in most cases, and is hardly noticeable in actual specimens, and our model is, after all, only a rough approximation to the ventricle. Its effect

might be appreciable in cases of very large areas of damage, and in these, because it is neglected, the bulges should be slightly larger than calculated.

The only data required for this investigation, other than geometrical, are some aspects of the tension-length relation for cardiac muscle in isometric contraction. At first some calculations were made assuming that the extension of the muscle fibres due to a bulge causes no increase in their tension; this would be a permissible approximation in small bulges, for which the extra length of the fibres is relatively small. The results so obtained were, however, superseded, and are not reported in this paper. In appreciable bulges the extra length of the muscle fibres might be expected to entail very substantial increases of tension.

Quantitative information on the tension-length relation for human cardiac muscle is not available, but an estimate of its behaviour in isometric contraction is made on the bases of Lundin's observations on frog's cardiac muscle and of Evans and Hill's (1914–15) measurements on frog's skeletal muscle. The main relevant feature of these observations is that the *total* tension developed is a linear function of the length at which contraction occurs.[2] Such a linear relation is accordingly assumed for human cardiac muscle. One other assumption, concerning the length at which zero tension would occur on contraction, is made and is discussed in the section headed "Tension-length relation".

An assumption has to be made regarding the extent to which the increased tension in a bulge in an intact layer is distributed throughout the remaining more or less spherical portion of the layer. A fairly uniform distribution might be expected if the various muscle layers could slide freely over one another; the tension in the layer would then be determined by the fractional increase in length of the entire muscle fibre in the layer. However, it is thought from the general structure of the wall that only a little sliding of layers can occur, and this prevents any substantial increase of length and so of tension except in the bulge. It is, therefore, assumed that the tension in the bulging portion of a layer is determined by the fractional increase in length of only that part of the muscle in the bulge while the tension in the remaining portion of the layer is assumed to be the same as in the absence of damage.

The muscle layers in the region of an infaret are supposed to have the same thicknesses as in an undamaged ventricle, even where a bulge causes considerable stretching. This convenient, though perhaps rather rough approximation, is probably adequate, as the cases in which it is far wrong are those in which there is considerable stretching and this only occurs in layers which are already very thin.

There is scarcely any experimental information on the relative values of the tensions in the various layers of an undiseased ventricle. There is also no indisputable mechanical reason why these tensions should be related at all, since membranes with different tensions can be kept in equilibrium with the same curvature by different pressure gradients. However, the most probable arrangement is that the tensile stress (tension per unit area of cross-section) should be approximately the same in each layer, since to be most effective in pumping it should be as large as the structure of muscle permits. This is accordingly assumed and is supported by the observations of Lowe and Bate that the fibre size is normally uniform throughout all the ventricular muscles.

The tension in the walls at the moment considered is such as to be in equilibrium with the diastolic arterial pressure of blood within and it is assumed that this pressure is the same whether or not infarction is present. The internal capacity at the phase considered is 65 ml., except for hypertrophied hearts for which the corresponding capacity is taken to be 240 ml.: these figures determine the radii of the muscle layers.

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[2] This feature was not mentioned by Evans and Hill who were interested in the *increase* of tension occurring on contraction.

ANALYSIS.

Preliminary Formulae.

(i) If a uniform flexible membrane is in equilibrium in the shape of part of a spherical shell with inner and outer radii r_1 and r_2, when subject to uniform inner and outer pressures p_1 and p_2 and uniform tensile stress T round the edge, then

$$r_1^2 p_1 - r_2^2 p_2 = (r_2^2 - r_1^2)T. \tag{1}$$

This result may be obtained by considering the equilibrium of a small piece of the membrane, like the well-known relation between pressure and tension in a bubble; but for (1) the thickness of the membrane is not restricted to be small.

(ii) The volume cut off from a sphere of radius R by a plane whose circle of section subtends an angle 2ψ at the centre of the sphere is

$$\frac{\pi R^3}{12}(8 - 9\cos\psi + \cos 3\psi). \tag{2}$$

Notation.

Let a^1 and a be the radii of the inner intact layer, and b and b^1 be the radii of the outer intact layer; thus a and b are the inner and outer radii of the middle layer in the region where it is not damaged (see Fig. 1).

Let r and s be the inner and outer radii of the liquid infarct between the inner and outer layers (see Fig. 1).

Let r^1 and s^1 be the inner and outer radii of the whole wall at the infarct; so that, by the assumption of unaltered thickness of the layers where they are stretched,

$$r - r^1 = a - a^1, \qquad s^1 - s = b^1 - b. \tag{3}$$

Let 2ω be the angle subtended at the centre by the infarct (see Fig. 1).

Let 2θ and 2ϕ be the angles subtended, at their respective centres of curvature, by the inner and outer surfaces of the liquid infarct.

Let p be the pressure inside the ventricle at the moment considered, and p^1 the corresponding pressure in the liquid of the infarct. These are, of course, excess pressures over that on the external surface.

Let T be the uniform circumferential tensile stress in all layers in the undamaged ventricle, at the moment considered.

Let T_θ and T_ϕ be the tensile stresses in the inner and outer layers where they contain the infarct.

Equations of Equilibrium and Their Reduction.

For equilibrium of the undamaged ventricle, applying (1),

$$a^{1^2} p = (b^{1^2} - a^{1^2})T. \tag{4}$$

For equilibrium of the inner and outer bulges of the damaged ventricle, applying (1) to each,

$$r^{1^2} p - r^2 p^1 = (r^2 - r^{1^2})T_\theta, \tag{5}$$

$$s^2 p^1 = (s^{1^2} - s^2)T_\phi. \tag{6}$$

In reducing these equations the aim is to express all the unknown quantities in terms of the unknown angles θ and ϕ. The equations finally obtained for these unknowns are (9) and (10), which essentially express the condition of equilibrium in the bulging wall and the condition of unchanged volume of the liquid within it, respectively.

The result of eliminating p and p^1 from (4)–(6) can be written

$$\left(\frac{r^1}{r}\cdot\frac{b^1}{a^1}\cdot\frac{s}{s^1}\right)^2=1+\left(\frac{r^1s}{rs^1}\right)^2\left(\frac{r^2}{r^{12}}-1\right)\left(\frac{T_\theta}{T}-1\right)+\left(1-\frac{s^2}{s^{12}}\right)\left(\frac{T_\phi}{T}-1\right).$$

Now the right-hand side is approximately 1, the other terms being small because r/r^1 and s/s^1 are approximately 1 and T_θ and T_ϕ do not rise to more than a few times T. Hence approximately

$$\begin{aligned}\frac{r^1}{r}\cdot\frac{b^1}{a^1}\cdot\frac{s}{s^1}&=1+\frac{1}{2}\left(\frac{r^1s}{rs^1}\right)^2\left(\frac{r^2}{r^{12}}-1\right)\left(\frac{T_\theta}{T}-1\right)+\frac{1}{2}\left(1-\frac{s^2}{s^{12}}\right)\left(\frac{T_\phi}{T}-1\right)\\&=1+\frac{1}{2}\left(\frac{a^1}{b^1}\right)^2\left(\frac{r^2}{r^{12}}-1\right)\left(\frac{T_\theta}{T}-1\right)+\frac{1}{2}\left(1-\frac{s^2}{s^{12}}\right)\left(\frac{T_\phi}{T}-1\right)\\&=1+\frac{a^{12}}{b^{12}}\left(\frac{r}{r^1}-1\right)\left(\frac{T_\theta}{T}-1\right)+\left(1-\frac{s}{s^1}\right)\left(\frac{T_\phi}{T}-1\right);\end{aligned}$$

and because the terms after the first on the right are small,

$$\frac{1}{a^1}\cdot\frac{r^1}{r}\cdot\left\{1-\left(1-\frac{s}{s^1}\right)\left(\frac{T_\phi}{T}-1\right)\right\}=\frac{1}{b^1}\cdot\frac{s^1}{s}\cdot\left\{1+\frac{a^{12}}{b^{12}}\left(\frac{r}{r^1}-1\right)\left(\frac{T_\theta}{T}-1\right)\right\}.$$

Applying (3), this becomes

$$\frac{1}{a^1}\left\{1-\frac{a-a^1}{r}\right\}\left\{1-\frac{b^1-b}{s^1}\left(\frac{T_\phi}{T}-1\right)\right\}=\frac{1}{b^1}\left\{1+\frac{b^1-b}{s}\right\}\left\{1+\frac{a^{12}}{b^{12}}\cdot\frac{a-a^1}{r^1}\left(\frac{T_\theta}{T}-1\right)\right\},$$

and r^1 and s^1 can be finally eliminated by using the approximation of r/r^1 and s/s^1 to 1 and neglecting second order terms in the small ratios $(a-a^1)/r$ and $(b^1-b)/s$:

$$\frac{1}{a^1}\left\{1-\frac{a-a^1}{r}-\frac{b^1-b}{s}\left(\frac{T_\phi}{T}-1\right)\right\}=\frac{1}{b^1}\left\{1+\frac{b^1-b}{s}+\frac{a^{12}}{b^{12}}\,\frac{a-a^1}{r}\left(\frac{T_\theta}{T}-1\right)\right\}. \tag{7}$$

Without further approximations, but using the substitutions

$$r=a\,\frac{\sin\omega}{\sin\theta},\qquad s=b\,\frac{\sin\omega}{\sin\phi}, \tag{8}$$

which are clear from Fig. 1, equation (7) becomes

$$\left(\frac{1}{a^1}-\frac{1}{a}\right)\left\{1+\frac{a^{13}}{b^{13}}\left(\frac{T_\theta}{T}-1\right)\right\}\sin\theta+\left(\frac{1}{b}-\frac{1}{b^1}\right)\left\{1+\frac{b^1}{a^1}\left(\frac{T_\phi}{T}-1\right)\right\}\sin\phi=\left(\frac{1}{a^1}-\frac{1}{b^1}\right)\sin\omega. \tag{9}$$

The condition that the liquid-filled region in the bulge has the same volume as the muscle which it replaces is easily written down by four applications of (2), and can readily be rewritten, without approximations, in the form

$$\begin{aligned}&\frac{b^3}{\sin^3\phi}\,(8-9\cos\phi+\cos3\phi)-\frac{a^3}{\sin^3\theta}\,(8-9\cos\theta+\cos3\theta)\\&\qquad=\frac{b^3-a^3}{\sin^3\omega}\,(8-9\cos\omega+\cos3\omega).\end{aligned} \tag{10}$$

Equations (9) and (10) are sufficient to determine θ and ϕ, given a^1, a, b, b^1 and ω, if the "tension factors" T_θ/T and T_ϕ/T are expressible in terms of these quantities. This requires some knowledge of the tension-length relation for the muscle fibres concerned, and is discussed in the next section, the expressions obtained being (11) and (12).

Equations (9) and (10), using (11) and (12), were solved numerically in a number of

cases, giving the results tabulated. The "height factors" of the bulges are calculated from the solutions for θ and ϕ by the formulae:

$$\left.\begin{aligned}\text{Height factor of inner bulge} &= \cot \tfrac{1}{2}\omega \tan \tfrac{1}{2}\theta \\ \text{Height factor of outer bulge} &= \cot \tfrac{1}{2}\omega \tan \tfrac{1}{2}\phi\end{aligned}\right\} \quad (13)$$

These are obtained as follows, referring to Fig. 1:

Height of cap of inner bulge: undistorted height

$$= \frac{r(1-\cos\theta)}{a(1-\cos\omega)} = \frac{\sin\omega}{\sin\theta} \cdot \frac{1-\cos\theta}{1-\cos\omega} = \cot\tfrac{1}{2}\omega \tan\tfrac{1}{2}\theta$$

using (8); and similarly for the outer bulge.

TENSION-LENGTH RELATION.

The preceding analysis involves no assumption as to the relation between tension and extension of the distorted parts of the undamaged muscle layers. Some aspects of this relation are now required[3] in order to express the tension factors T_θ/T and T_ϕ/T for the inner and outer bulges in terms of θ and ϕ, which are then the only unknowns in equations (9) and (10).

Lundin gives a tension-length graph for frog's cardiac muscle in isometric contraction, which shows that the total tension of the contracted muscle is practically a linear function of the length over the whole range from resting length to rupture.

Evans and Hill tabulated the *increase* of tension developed in frog's skeletal muscle on isometric contraction, and also the *initial* tension before contraction. If these data are added and a graph drawn, it is found that the total tension in isometric contraction is again practically a linear function of the length.

We assume, then, that the tension in human cardiac muscle is a linear function of the length. Consideration of a graph shows that the tension is then proportional to the "excess" of length, this "excess" being measured from the hypothetical state in which the muscle exerts zero tension on contraction.

The ratio of tensile stresses in the bulged and normal states of the outer layer is thus equal to the ratio of "excess" lengths of muscle fibres crossing the bulge, so that

$$\frac{T_\phi}{T} = \frac{2s\phi - 2b_o\omega}{2b\omega - 2b_o\omega}$$

where b_0 is the radius the outer layer would have if it were permitted to shrink to the state of exerting zero tension on contraction. It is assumed for simplicity that this layer remains spherical. The thickness of the layer is disregarded. It now follows that

$$\frac{T_\phi}{T} - 1 = \left(\frac{s\phi}{b\omega} - 1\right) \Big/ \left(1 - \frac{b_o}{b}\right) = \left(\frac{\phi \sin\omega}{\omega \sin\phi} - 1\right) \Big/ \left(1 - \frac{b_o}{b}\right) \quad (11)$$

using (8). Similarly for the inner layer

$$\frac{T_\theta}{T} - 1 = \left(\frac{\theta \sin\omega}{\omega \sin\theta} - 1\right) \Big/ \left(1 - \frac{a_o}{a}\right) \quad (12)$$

where a_0 is the radius the inner layer would have if spherical and exerting zero tension on contraction.

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[3] The authors realize that this discussion of the tension-length relation involves somewhat hypothetical assumptions. However, this objection does not apply to the analysis of the preceding section, which can be applied whatever tension-length relation is assumed.

The estimation of values of a_o and b_o is rather hazardous. The view we have taken is that both b_o/b and a_o/a are the ratio of length of muscle for zero tension on contraction to length of muscle for normal tension at the phase considered, so that they should be the same:

$$\frac{b_o}{b}=\frac{a_o}{a}.$$

Now to estimate this ratio, we notice that, in Lundin's tension-length curve and also in that constructed from Evans and Hill's data, the extrapolated length for zero tension on contraction is 0·86 times the resting length (i.e. length for zero tension before contraction). We assume that the same holds for human cardiac muscle. Consequently, b_o is 0·86 times the radius of the outer layer, supposed spherical for simplicity, when the ventricle relaxes with zero internal volume, having expelled its contents. In this state the outer surface encloses a volume equal to the volume of muscle composing the ventricle, 130 cm.^{3} as calculated from the geometrical data, so that its radius is 3·15 cm. Thus

$$b_o = 0{\cdot}86 \times 3{\cdot}15 = 2{\cdot}7,$$

and so

$$\frac{a_o}{a}=\frac{b_o}{b}=\frac{2{\cdot}7}{3{\cdot}6}=\frac{3}{4}.$$

Magnitude of Muscle Tensions.

Upper limits to the tension factors T_θ/T and T_ϕ/T, which are the ratios of tensions in the bulged layers to the corresponding tensions in undamaged hearts, can be estimated on the basis of Lundin's results.

The outer surface of the ventricle in the relaxed state is of radius 3·15 cm., as above, while at the systolic phase considered its radius is 3·6 cm. Thus at this phase the length of its constituent fibres is 1·14 times the resting length; the corresponding tension on Lundin's graph is 40 units. The muscles used by Lundin snapped at lengths between 2·25 and 2·50 times the resting length, and the corresponding tensions range from 200–300 units. Thus rupture of the ventricular wall might be expected at tension factors between $200/40 = 5$ and $300/40 = 7{\cdot}5$.

Lundin's work refers to frog's cardiac muscle. For human cardiac muscle some information as to the magnitude of the tension factors can be obtained as follows.

The relation between the internal pressure p in the human ventricular cavity and the tensile stress T in the wall is given in (4) of p. 502. This may be restated

$$\frac{p}{T}=\left(\frac{b^1}{a^1}\right)^2-1$$

Here p represents the diastolic pressure, which is taken to be 80 mm. Hg. at average stroke volume (65 ml.) and 85 mm. Hg. at maximum stroke volume (180 ml.).

At average output

$$\frac{p}{T}=\left(\frac{3{\cdot}6}{2{\cdot}5}\right)^2-1=1{\cdot}07$$

Similarly at maximum output

$$\frac{p_{\max}}{T_{\max}}=\left(\frac{4{\cdot}2}{3{\cdot}5}\right)^2-1=0{\cdot}44$$

Therefore at average output

$$T = p/1{\cdot}07 = 80/1{\cdot}07 = 75 \text{ mm. Hg.}$$[4]

while at maximum output

$$T_{max} = p_{max}/0{\cdot}44 = 85/0{\cdot}44 = 193 \text{ mm. Hg.}$$

The ratio of maximum tensile stress to average tensile stress is

$$\frac{T_{max}}{T} = \frac{193}{75} = 2{\cdot}6$$

This ratio indicates that tension factors of the order of 2·6 commonly exist in the human heart under normal physiological conditions. It does not, however, indicate their maximum values. Lundin's observations on frog's cardiac muscle show that in that animal the maximum tension factors lie between 5 and 7·5 (see above) and that tearing of the muscle is likely to occur at these levels.

ANEURYSMS IN HYPERTROPHIED HEARTS.

In a recent paper Lowe and Bate have presented measurements of the size of the cardiac muscle fibres in hearts at "the limit of hypertrophy". They found that these hypertrophied fibres had a cross-sectional area 2·4 times that of normal fibres. The various linear dimensions required in this analysis have, therefore, been multiplied by 1·55 ($= \sqrt{2{\cdot}4}$) to obtain values for hearts at "the limit of hypertrophy". The thicknesses of the four muscle layers in the left ventricle at the phase considered have been taken, therefore, as 1·5 mm., 9·5 mm., 4·5 mm., 1·5 mm. The internal ventricular capacity is 240 ml. and the volume of muscle is 480 ml.

This increase in volume of the ventricular cavity is presumed because if it is assumed spherical then such increase in surface of the cavity is necessary to accommodate the enlarged fibres of the hypertrophied heart. It is also apparent that this implies that the ventricular cavity in these hypertrophied hearts does not empty in systole.

For the tension-length relation we apply, for want of other information, the same assumptions as used previously, namely that the graph is linear and that zero tension occurs at 0·86 times the resting length. We also assume that the stroke volume of the hypertrophied ventricle is the same as for the normal ventricle, which we have taken to be 65 ml. Then the internal capacity of the hypertrophied ventricle in the relaxed state during diastole is (240–65) ml. = 175 ml. Adding the volume of muscle we obtain the corresponding external radius 5·4 cm. As before, b_0 is taken as 0·86 of the radius in the relaxed state, so that

$$b_0 = 0{\cdot}86 \times 5{\cdot}4 = 4{\cdot}64.$$

Thus

$$\frac{a_o}{a} = \frac{b_o}{b} = \frac{4{\cdot}64}{5{\cdot}55} = 0{\cdot}84.$$

This value is now used for substitution in equations (11) and (12) in the case of hypertrophied hearts.

---

[4] Tensile stress is here expressed in the same units as pressure. This is an unusual unit to use but both pressure and tensile stress are forces, in the one case positive and in the other negative, acting per unit area, and hence can be expressed in the same units.

RESULTS.

The predicted deformities of the left ventricular wall following infarcts in the D.B.S. or D.S.S. muscles, or in both, subtending various angles at the centre of the ventricle are set out in the following Tables. The results are given

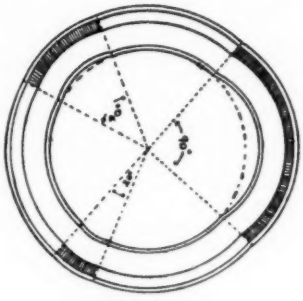

Fig. 3.

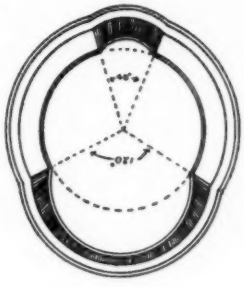

Fig. 4.

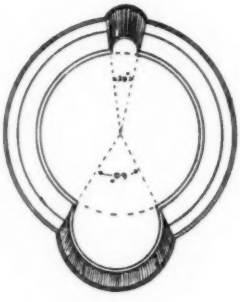

Fig. 5.

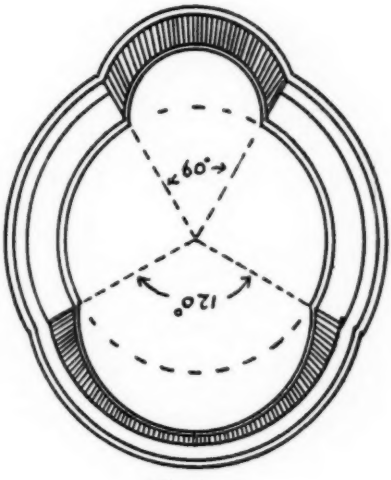

Fig. 6.

Fig. 3. Drawing of the calculated deformities of the ventricular wall due to infarcts in D.S.S. muscle.

Fig. 4. Drawing of the calculated deformities of the ventricular wall due to infarcts in D.B.S. muscle.

Fig. 5. Drawing of the calculated deformities of the ventricular wall due to infarcts in both D.S.S. and D.B.S. muscles.

Fig. 6. Drawing of the calculated deformities of the ventricular wall due to infarcts in a hypertrophied ventricle (cf. Figs. 4 and 5).

in terms of "Height Factors" (see page 500) for the inner and outer surface of bulge in the damaged layer and as "Tension Factors" (see page 503) for the intact layers on the inner and outer surface of the infarct. In Figs. 3–6 these deformities are shown in approximately half-scale drawings of the idealized ventricle.

TABLE 1.

(See Fig. 3).

Damage to D.S.S. muscle (3 mm. layer). Heart otherwise normal.

Angle of damage.	Height factor: Inner bulge.	Height factor: Outer bulge.	Tension factor: Inner bulge.	Tension factor: Outer bulge.
20°	1·29	1·21	1·01	1·01
40°	1·29	1·21	1·05	1·04
90°	1·28	1·22	1·24	1·18

TABLE 2.

(See Fig. 4).

Damage to D.B.S. muscle (6 mm. layer). Heart otherwise normal.

Angle of damage.	Height factor: Inner bulge.	Height factor: Outer bulge.	Tension factor: Inner bulge.	Tension factor: Outer bulge.
40°	2·69	1·94	1·48	1·22
80°	2·28	1·74	2·19	1·60
120°	2·01	1·60	2·74	1·95

TABLE 3.

(See Fig. 5).

Damage to D.S.S. (3 mm.) and D.B.S. (6 mm.) muscles. Heart otherwise normal.

Angle of damage.	Height factor: Inner bulge.	Height factor: Outer bulge.	Tension factor: Inner bulge.	Tension factor: Outer bulge.
20°	6·13	3·27	1·70	1·19
60°	5·51	3·49	5·03	2·76
(60°)	(6·13)	(3·93)	(5·00)	(2·84)
80°	5·39	3·61	7·01	4·04

TABLE 4.

(See Fig. 6).

Hypertrophied heart.

Angle of damage and layers affected.	Height factor: Inner bulge.	Height factor: Outer bulge.	Tension factor: Inner bulge.	Tension factor: Outer bulge.
120° of D.B.S. muscle	1·71	1·42	2·80	1·97
60° of D.B.S. and D.S.S. muscles	4·32	2·71	5·08	2·62

It is noted from the figures that the biggest calculated deformities are smaller than is observed in actual specimens. The authors consider that this indicates that the tension-length relationship for human cardiac muscle may be qualitatively the same as for frog's cardiac muscle but quantitatively is a little different. For instance, if the length for zero tension on contraction instead of being 0·86 times the resting length of the muscle were 0·80 times the resting length, the second result in Table 3 has the values set out in brackets. This means that for a small change in the properties of the muscle there would be a considerable increase in the calculated deformity.

DISCUSSION.

A study of the results obtained from this analysis shows that the two important factors determining the distortion produced in the wall of the ventricle are the proportion of the thickness of the wall destroyed and the size of the area of destruction. An increase of either of these factors produces a greater deformation, although increase of thickness of the damaged tissue has a greater effect than increase of lateral extent (represented by the angle subtended at the centre of the ventricle). Although the results are presented in terms of the muscles involved, it is obvious that this is only a translation into anatomical terms of the proportion of the wall thickness damaged. The presentation of the results in terms of the muscles involved is desirable because of the common involvement of full thickness of individual muscles in the naturally occurring disease.

In the examples calculated the maximum tension factors are of the order of the expected maximum values of 5 to 7·5. However, in cases of infarcts involving both D.B.S. and D.S.S. muscles subtending angles greater than 60°, the tension factor rises rapidly with increasing angle and rupture of the ventricle wall from tearing of the remaining muscle is to be expected. This deduction is in accord with the rarity with which large ventricular aneurysms subtending an angle greater than 60° are observed. It seems probable therefore that infarction of two deep muscles or, 85 p.c. of the thickness of the ventricular wall, subtending an angle greater than 60° will lead to rupture of the ventricular wall.

Before comparing the theoretical results with actual observed cases it is profitable to consider the changes introduced when the "liquid" infarct is replaced by mature fibrous tissue. Mechanically the damaged area, considered to have no tensile strength, is replaced by a tissue which has increasing tensile strength and which gradually dehydrates and diminishes considerably in volume. This scarring increases the strength of the wall and the dehydration produces greater thinning of the wall. The combined effects of these changes will be to bring the outer and inner surfaces of the ventricular wall nearer to the scar, which will itself tend to move inwards. The internal deformity of the ventri-

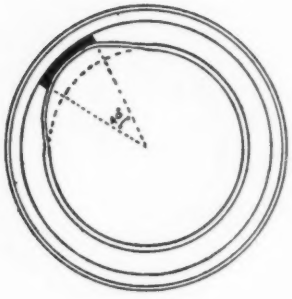

Fig. 7.

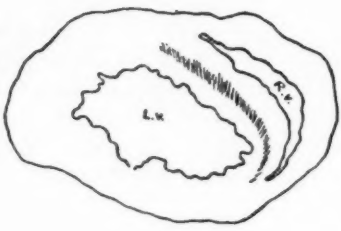

Fig. 8.

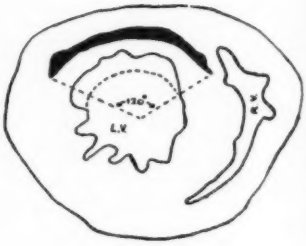

Fig. 9.

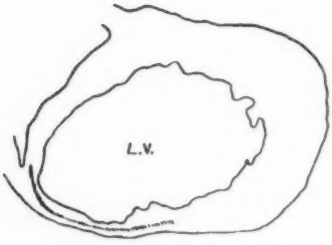

Fig. 10.

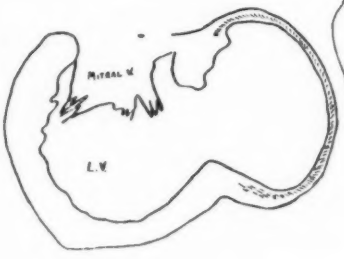

Fig. 11.

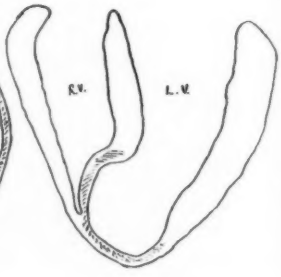

Fig. 12.

Fig. 7. Estimate of the late result of an infarct in D.B.S. muscle after scar formation (cf. Fig. 4).

Fig. 8. Drawing of a section of a heart with a recent infarct in D.S.S. muscle subtending an angle of 90° (cf. Fig. 3).

Fig. 9. Drawing of a section of a heart with an old infarct in D.B.S. muscle subtending an angle of 120°.

Fig. 10. Drawing of a section of a left ventricle with an infarct scar subtending an angle of 90°.

Fig. 11. Drawing of a section of a left ventricle with infarct scars in D.B.S. and D.S.S. muscles subtending an angle of 60° (cf. Fig. 5).

Fig. 12. Drawing of a section of a heart with a recent infarct involving 85 p.c. of wall thickness at apex and subtending an angle of 40° (cf. Fig. 5).

cular cavity will therefore increase. By contrast the external deformity will diminish, partly because of the indrawing action of the dehydrating fibrous tissue and partly because of the interposition between the outer and inner layers of the wall of a new layer with considerable tensile strength (cf. Figs. 4 and 7).

It will be appreciated therefore that the deformity of the wall following infarction will be at its maximum during the liquid phase of the infarction and will not increase externally beyond that point. This is in contrast to the view previously expressed by one of us (Lowe and Wartman, 1944) that the fibrous scar gradually stretched as is assumed to occur in aneurysm of the aorta. The

difference between the two conditions is due to the nature of the underlying pathological processes. In the aorta there is continual destruction of the new-formed fibrous tissue by the syphilitic lesion, whereas in cardiac aneurysms due to infarction there is not this continuing destruction of fibrous tissue.

The scale drawings (Figs. 3, 4, 5, 6) of the theoretical deformations of the ventricular wall show that with infarcts in the D.S.S. muscle (25 p.c. of the wall thickness) with angles up to 120° there is no gross external deformity of the ventricle produced. With the larger angles there is some external deformity which might be appreciated by the naked eye at post-mortem examination, but it would not be detected by radioscopy during life. Even in these cases, when scarring of the infarct has occurred, the external deformity would be expected to disappear. Fig. 8 is a drawing made from a case of recent infarction in the D.S.S. muscle subtending an angle of some 90° and there is very little deformity, internal or external, noticeable.

In the case of infarctions involving the D.B.S. muscle (50 p.c. of wall thickness) at small angles no external deformity is expected to be detectable clinically, but with big angles this might be possible in the acute phase. However, when scarring has occurred the external deformity would be reduced and probably not seen by radioscopy. In these latter cases there will be gross thinning of the ventricular wall and deformity of the ventricular cavity. This is well illustrated in Figs. 9 and 10, drawn from cases of long-standing infarction in D.B.S. muscle subtending angles of 120° and 90° respectively.

When both D.B.S. and D.S.S. muscles (85 p.c. of wall thickness) are involved at the same site, then definite external deformities of the wall occur even at small angles. With angles of the order of 60° large aneurysmal bulges develop, and persist after the scarring occurs. Fig. 11 shows the late result of infarction of both D.B.S. and D.S.S. muscles subtending an angle of 60°. There is a well-developed aneurysmal sac opening off the main ventricular cavity.

It is only in the upper three-fourths of the left ventricular wall that the deep muscles are situated. The wall at the apex, which has much the same thickness as elsewhere, is composed solely of the intertwining superficial muscles. Similar considerations apply in this apical region and with involvement of about 85 p.c. of the wall thickness in an infarct an aneurysmal sac will develop. This is illustrated in Fig. 12 in which a recent apical infarct, involving about 85 p.c. of the wall thickness over an arc of 40°, has produced an appreciable aneurysm in the interventricular septum.

This analysis shows that there is no essential difference in the mechanisms which lead to scarring and thinning of the ventricular wall after myocardial infarction and those which lead to the formation of aneurysms. From the clinical viewpoint, however, distinction between the two types is valuable. When only internal deformity and thinning of the wall occur, thrombosis on the endocardium over the deformity is unlikely, for the contours of the deformity are

gradual. With aneurysm formation clotting of blood in the sac is common and the risks of thrombo-embolism are introduced into the clinical picture and prognosis.

From this discussion the following conclusions may be drawn. First, that infarcts of the D.S.S. muscle alone will produce some thinning of the left ventricular wall, but are unlikely to lead to aneurysm formation even when extensive. Secondly, infarcts of the D.B.S. muscle will produce a greater thinning of wall but no aneurysm formation, except perhaps when they subtend very large angles. Thirdly, infarcts involving both D.B.S. and D.S.S. muscles at the same site will normally lead to aneurysm formation unless they are of very small extent. If they subtend angles much in excess of 60°, they will probably lead to ventricular rupture. Fourthly, infarcts in the superficial muscles will produce no real disturbance in the ventricular wall unless they occur in the apical fourth of the heart and then their effects may be any of those already considered, depending on the thickness of the wall involved.

It is to be noted that these conclusions apply to the left ventricular wall. The right ventricular wall is composed of portions of the superficial and D.S.S. muscles only, and whilst the general principles enunciated here are applicable to it, no examples have been calculated, as aneurysm formation in the right ventricle is very rare.

An interesting extension of this analysis is the consideration of two instances of infarcts occurring in hypertrophied hearts. It is seen from Fig. 6 that the distortions calculated are slightly less than in the case of normal heart muscles, but of the same general types. Further, it is seen that although the distortions are less, the tension factors are of the same order as in non-hypertrophied hearts and therefore it is unlikely that the above conclusions about maximum sizes of infarcts would be upset.

The conclusions drawn from this study would have an important practical application if there were a reliable clinical method for determining the muscles involved and the extent of damage in those muscles in cases of infarction. Electrocardiography, as yet, is not able to supply this information and radioscopy is of value only in determining external cardiac deformity. If such methods were available, then the immediate prognosis of a case of myocardial infarction would be removed to some extent from statistical appraisal, for it would be possible to assess the probability of the following events developing: thinning of the ventricular wall only; aneurysm of the ventricle with the risk of thrombo-embolism; cardiac rupture. Such information would also influence the late prognosis, but this is determined more by the state of the coronary arteries than by the muscular destruction.

Finally, the good qualitative correspondence between the theoretical results and the observed natural cases indicates that the various assumptions which we

have made about the anatomy of the ventricle and the physiology of cardiac muscle are probably substantially correct.

SUMMARY.

A mechanical analysis of the formation of cardiac aneurysms is presented. This is based on the established anatomical structure of the ventricular wall, the properties of cardiac muscle and the physical nature of infarcted cardiac muscle.

It is shown that the development of a cardiac aneurysm is only a special case of the general problem of infarction in cardiac muscles.

Comparison of the theoretical deformations of the ventricular wall with those occurring naturally is good and indicates that the assumptions made were substantially correct.

A general conclusion is reached as to which types of myocardial infarction are likely to lead to aneurysm formation.

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OBSERVATIONS ON THE LIFE-CYCLE OF *PLASMODIUM MALARIAE*

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Quartan malaria occurs in native populations throughout the tropics, but its incidence, even in infants and young children, is often low. It is remarkable for its persistence in the human host, and for its slow development in both hosts. The experimental study of the disease has been hampered by the irregular appearance and paucity of gametocytes, and it is only within recent years that it has been carried through successive mosquito-man passages in a laboratory (de Buck, 1935; Kligler and Mer, 1937; Boyd, 1940; Young and Coatney, 1941). As far as we are aware, except for Heydon's (1923) experiment, no work with a Melanesian strain of *P. malariae* had been reported prior to the observations recorded below.

We had the opportunity of transmitting *P.malariae* to one volunteer, and of observing two trophozoite-induced infections derived by subinoculation, while we were members of the Entomological team of the L.H.Q. Medical Research Unit (A.I.F.), Cairns, North Queensland. The strain of parasites was obtained by S/Sgt. J. W. McNamara and Sgt. J. H. Brockhurst of the new Guinea detachment of the Unit, who succeeded in feeding a few bred *Anopheles punctulatus punctulatus* on a native child with *P. malariae* gametocytes in the blood. These mosquitoes were flown to Cairns, where they ultimately set up a pure quartan infection.

EXPERIMENTAL.

THE CYCLE IN THE MOSQUITO.

After 16 days' incubation, two of which were spent travelling and the remainder at a mean temperature of 73° F. (22·8° C.), the cysts were about 25μ in diameter (minimum 21·7μ, maximum 31·0μ). Ten days later, in another mosquito, cysts of approximately the same size and stage of development were found. Growth was evidently not only slow, but very variable. The pigment within the few cysts examined was concentrated in a double line of rather fine, black granules, an arrangement not seen in *P. vivax* or *P. falciparum*. The number of cysts varied from 2–10. No dissections were done from the 26th until the 31st day from feeding, when a light infection of the salivary glands was seen in a mosquito. The incubation in the mosquito, then, was of the order of 4 weeks, as compared with 15–16

days in *P. vivax*, and 18–20 days in *P. falciparum* (concurrent batches). The sporozoites measured between 13 and 14μ in length, being longer and also slightly thicker than those of *P. vivax* or *P. falciparum*.

All workers agree that *P. malariae* develops more slowly than either *P. vivax* or *P. falciparum*, but possibly at high temperatures the difference may not be very great. In Rabaul, Heydon (1923) found sporozoites in *A. punctulatus* 10 days after being fed on a native child with *P. malariae* gametocytes, although he considered the possibility that the child may have had a mixed infection somewhat invalidated his experiment. In Calcutta, Iyengar (1933) found complete development in *A. stephensi*, also in 10 days in one experiment in March, but in other experiments between 2 and 3 weeks were required. De Buck (1935) obtained complete development in 15 days at 27° C. (80·6° F.). Kliger and Mer (1937) found that 27 days were required at 24° C. (75·2° F.)—a figure fairly comparable with ours.

THE CYCLE IN MAN.

Infection and the Incubation Period.

Twenty-four days after feeding on the native child, the surviving mosquitoes (5 in number) engorged on a volunteer. Subsequently they were given the opportunity to bite him again at intervals until they died. In all he received 11 bites from 5 mosquitoes, 3 of which proved to be harbouring sporozoites when they came to dissection. The bites were spread over a period of 10 days, so that the exact incubation period could not be determined. Trophozoites were detected 24 days after the first bite and 14 days after the last one, but the parasites did not begin to build up to a level sufficient to cause symptoms until 2 weeks later (Fig. 1).

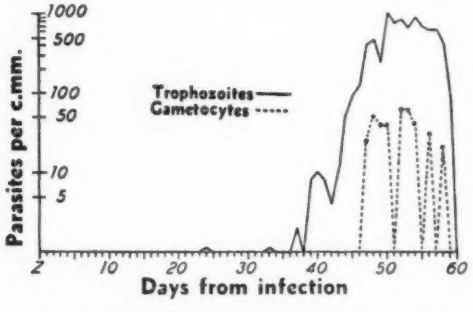

Fig. 1. *P. malariae*. Trophozoite and gametocyte waves in sporozoite-induced infection in W.S., bitten by 3 infected mosquitoes between day 2 and day 10.

Boyd (1940) recorded periods from infection to detection of parasites varying from 28–37 days in 5 patients naturally infected with North American strains of *P. malariae*. He also noted a lag of 3–12 days between the first detection of parasites and the onset of the clinical attack. De Buck (1935) infected four subjects, and observed periods of 23–26 days between biting and the detection of parasites. The number of bites given varied from 1–7. It seems likely, then, that the incubation period in our patient was nearer 24 than 14 days, and that he may have been infected at the first biting session.

The Trophozoite Wave.

The slow and irregular build up, with early remissions, is seen in Figs. 1 and 2. In W.S., the trophozoites reached a density of 1,020 per c.mm. on the 50th day, and then diminished slightly; they did not again reach the 1,000 per c.mm. level before treatment began on the 56th day. In R.R.H. (trophozoite-induced infection), the maximum count reached was 2,300 per c.mm. on the 67th day, just as therapy began (Fig. 2).

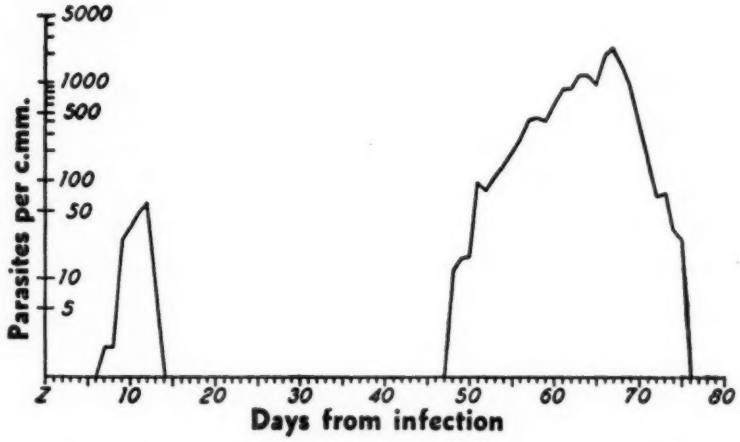

Fig. 2. *P. malariae.* Trophozoite wave in R.R.H., infected by the intravenous inoculation of 20 ml. blood from W.S. containing $1{\cdot}8 \times 10^6$ trophozoites.

In the third patient, F.S., the count never exceeded 400 per c.mm., although the infection was allowed to persist for 98 days before treatment began. At the beginning of this infection, the trophozoite density increased about 6 or 7 times at each of the first 3 schizogonies; but, from then until the 98th day, destruction of parasites just kept pace with their multiplication, and the count fluctuated about the 200 per c.mm. level. In each 72-hour period, the count would fall slightly, and then rise again following schizogony, but neither host nor parasite was able to gain any advantage (Fig. 3).

Our results are in agreement with those of many workers, who state that the densities attained by *P. malariae* are low in comparison with *P. vivax* and *P. falciparum*. The infections quoted by Boyd (1940), however, seem to have attained much higher parasite densities than ours. Boyd found that patients, whose attacks terminated spontaneously, had maximum

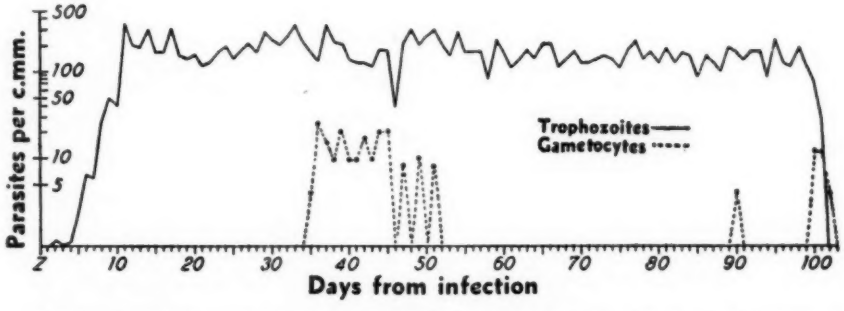

Fig. 3. *P. malariae.* Trophozoite and gametocyte waves in F.S., infected by intravenous inoculation of 400 ml. blood from R.R.H. containing $6{\cdot}4 \times 10^6$ trophozoites.

counts ranging from 3,000–12,500 per c.mm. Those who required therapy had much higher densities, and one hyperinfection with a maximum count of 114,000 per c.mm. was recorded.

Gametocyte Wave.

Gametocytes appeared sporadically and in small numbers in two of the three patients. In the sporozoite-induced infection, they were first seen 23 days after the first trophozoite, and 10 days after the beginning of the clinical attack. The maximum count recorded was 60 per c.mm., and all attempts to infect mosquitoes failed. In one trophozoite-induced infection, gametocytes were not seen until 33 days after the first trophozoites, and, although present for the succeeding 16 days, the maximum count recorded was only 24 per c.mm.

The poor gametocyte response in these patients was undoubtedly related to the low level of trophozoite densities. Most workers agree that gametocytes are infrequently produced and scanty in numbers, but Young and Coatney (1941) found them in all their infections. They considered, however, that the low absolute number formed was related to the low blood stream population of this species.

Morphology.

We made films at eight-hourly intervals from F.S., who had a typical quartan fever and a synchronous brood of parasites. The observations were begun immediately after a schizogony. The young rings grew slowly, very little change being apparent at the end of 8 hours. Larger rings and narrow band forms were present at 16 hours. Between 16 and 24 hours pigment granules became visible and the trophozoites were mainly amoeboid in shape. At 32 hours, pigment was abundant and broad band forms were seen. At 48 hours, the trophozoites almost filled the red-cells, growth being nearly complete. Occasionally two nuclei were present showing that the first division of the schizont nucleus had occurred. At 56 hours, there were usually 3–5 nuclei, and by the 64th hour usually 7–8 nuclei were present. At this time (64 hours) the pigment was becoming aggregated into coarse lumps. During the next 8 hours the merozoites were differentiated, and the pigment fused into a solid mass. The number of merozoites produced varied from 5–10, the average for 30 mature schizonts being 8·0. The most frequently observed number was 8, occurring in 30 p.c. of these schizonts. The long period required for the division of the schizont nucleus into 8 daughter nuclei is noteworthy, and may account for the frequency with which schizonts are seen in blood films of natives.

SUMMARY.

The experimental transmission of *Plasmodium malariae* by *Anopheles punctulatus punctulatus* is described. The mosquito cycle lasted about 4 weeks at a mean temperature of 22·8° C., compared with 15 to 16 days in *P. vivax* and 18 to 20 days in *P. falciparum* at the same time.

The period between the introduction of sporozoites and the detection of parasites in the blood lay between 14 and 24 days, but the trophozoites did not increase sufficiently to cause symptoms until another 2 weeks had elapsed. Gametocytes appeared 10 days after the beginning of the clinical attack. Both trophozoite and gametocyte densities remained low in all cases observed.

The rate of growth of the trophozoite, and formation of the schizont are described. The average number of merozoites formed was 8. A long period, nearly 24 hours, was required for the division of the schizont nucleus into 8 daughter nuclei.

Acknowledgments. We are indebted to Professor N. Hamilton Fairley, formerly Director of the L.H.Q. Medical Research Unit, for permission to publish, and to Dr. C. R. Bickerton Blackburn, former C.O. of the Unit, for designing the experiments.

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FIBRINOLYSIS FOLLOWING ELECTRICALLY INDUCED CONVULSIONS

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The application in this hospital of alternating current for electro-convulsive therapy (E.C.T.) and electronarcosis in certain mental disturbances afforded the opportunity of investigating the effects of this form of therapy on some of the components of the blood.

Schütz (1942) observed *in vitro* a delayed coagulation of blood following application of a weak direct current.

Brecht and Kummer (1943) noted a leucocytosis immediately after electrical shock; there was no alteration of blood sugar or protein.

Spiegel-Adolf, Spiegel *et al.* (1945) reported that following the application of a 60 cycle current at 30 volts for 1·2 seconds there were changes in cerebrospinal fluid suggesting the breakdown of nuclear substances. This statement, however, has since been contradicted by M. H. Hack (1947). Huddleson (1946) observed haemorrhages in the central nervous system in rats following electrical shock. Trojaniello (1947) indicated that dogs subjected to electrical shock by alternating current at 120 volts for a fraction of a second, showed no changes in blood lipase and diastase, but that phosphatase activity decreased for about 12 hours. Delay and Soulairac (1943) observed, following E.C.T. in human subjects, a transient increase in blood albumin, and occasionally a slight increase in the globulin fraction. There was a concomitant increase of blood calcium and inorganic phosphate. Further, hyperglycemia, leucocytosis, and a decrease in the alkali reserve were demonstrable.

EXPERIMENTAL.

Electrical convulsions were induced in suitable patients by alternating current (50 cycles) at 120 volts. Three to eight shocks, each of a duration of 0·1 seconds, were given. Blood samples were obtained by venepuncture before and after application of the current and mixed with one-ninth volume of 0·1 M sodium oxalate solution. The ratio of plasma: cell volume was determined in tubes of uniform size and shape and centrifugation was carried out under identical conditions, which should ensure comparable results, if not absolute values.

[1] Working under full-time grant from the National Health and Medical Research Council.

TABLE 1.

Influence of electrically induced shock on plasma cell ratio.

(Figures are given for plasma volume per 100 ml. of whole blood, and are corrected for oxalate addition).

	Before shock.	1–3 mins. after shock.	Change.
1	47·1	45·7	—1·4
2	52·8	50·8	—2·0
3	50·0	47·8	—2·2
4	54·6	51·2	—3·4
5	51·7	50·0	—1·7
6	44·3	44·3	0·0
7	48·2	47·7	—0·5
8	50·0	47·4	—2·6
9	48·9	44·8	—4·1
10	54·0	52·9	—1·1

The results indicate that the convulsions are followed by a slight haemo-concentration.

The Effect of E.C.T. on Plasma Proteins.

The estimations of total protein in oxalated plasma were carried out by a biuret technique similar to that given by Fantl and Nance (1947). Fibrinogen was determined by using 1 ml. of plasma to which were added 25 ml. of 0·15 M sodium chloride and 1 ml. of 0·25 M calcium chloride solution. After standing for 2–3 hours at 20° C. the fibrin was centrifuged and washed with saline until free of soluble proteins, and the estimations carried out by the biuret reaction.

TABLE 2.

Influence of electrical shock on concentration of plasma proteins.

No.	Before shock	After shock	P.c. change
1	1,250	1,309	+4·7
2	—	1,460 immediately after shock 1,351 40 min. after shock	+8·0
3	1,232	1,322	+7·0
4	1,257	1,327	+5·5
5	1,040	1,090	+5·0
6	1,155	1,172	+2·0
7	1,037	1,080	+4·0
8	1,275	1,375	+7·5
9	1,170	1,280	+8·5
10	1,148	1,172	+2·2

The figures represent mg. protein nitrogen per 100 ml. plasma, and are corrected for oxalate addition. They show a definite hyperproteinaemia following E.C.T.

Estimations of fibrinogen in the pre- and post-shock period did not show any variation greater than the limit of error of the technique, which is ± 3 p.c.

Prothrombin estimations carried out in whole plasma according to Quick's technique (1938) did not indicate any change in prothrombin activity when compared to the pre-shock level.

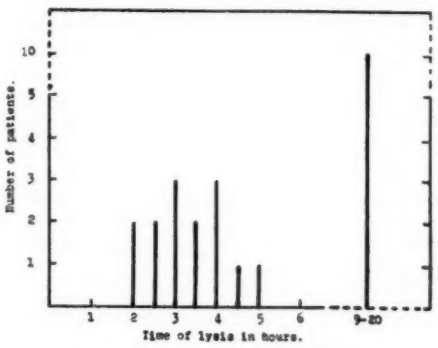

Fig. 1. Time of lysis of fibrin clot.

Effect of E.C.T. on Fibrinolysis.

The fibrinolytic activity of the plasma was determined by the following technique: to 0·1 ml. of oxalated plasma were added 2·4 ml. of 0·15 M sodium chloride containing 1 × 10–4 merthiolate, and 0·1 ml. of 0·25 M calcium chloride. The specimens were incubated at 37° C. and the time for complete disappearance of fibrin was determined. For the quantitative estimations 10 times greater volumes were used.

Dissolution of fibrin occurred in 24 out of 25 cases when blood was taken 1–3 minutes after convulsions. In the case in which no fibrinolysis was observed the patient obtained, 4 hours prior to E.C.T., 80 units of insulin. However, on repetition without pre-treatment with insulin, fibrinolysis in this patient occurred in 4 hours. Other patients obtaining insulin prior to E.C.T. showed fibrinolysis. As shown in Fig. 1, the time of fibrinolysis varied from 2 hours to approximately 19 hours. In some cases of extreme anxiety in the pre-shock state fibrinolysis occurred, and if it was of a minor degree it was enhanced following E.C.T. The phenomenon of fibrinolysis is only transient, since on several occasions in blood samples taken 40 minutes after application of the current no fibrinolysis could be observed whilst in blood samples taken 1 minute after the shock fibrinolysis occurred in 2 hours.

TABLE 4.

No. of experiment	Fibrinolysis in hours	Fibrinogen N concentration in fresh plasma	Fibrinogen N concentration in plasma incubated at 37° C.
1	3	44·7	43·0
2	18	49·0	47·0
3	18	67·2	68·5
4	18	60·3	66·0
5	3	54·1	50·0*
6	2¼	56·7	55·0*

Figures are given in mg. p.c. fibrinogen nitrogen, and are corrected for the addition of oxalate.

Experiments 5 and 6 were carried out on plasma from which prothrombin had been removed by alumina adsorption, and fibrin was produced by addition of thrombin.

In order to find out whether the proteolysis was confined to fibrin, oxalated plasma was incubated without calcium ions for 12 hours longer than was required for complete fibrinolysis in the presence of Ca ions. When to the former specimens calcium chloride was now added, fibrin formation took place, and an estimation indicated a complete recovery of fibrinogen, as can be seen from Table 4.

From these results it is evident that, in contrast to complete fibrin breakdown, fibrinogen was not affected.

The fibrinolytic enzyme is inactivated during prolonged incubation at 37° C. because fibrin produced by recalcification of oxalated plasma after 24 hours' incubation took considerably longer to disappear than it did when fresh plasma specimens were investigated.

In further experiments it could be shown that oxalate is not an inhibitor of this system, because following the addition of thrombin without calcium ions, fibrinolysis occurred in the same period as in the presence of calcium ions. In addition, it appears that calcium ions are not essential for fibrinolysis. Experiments were carried out in which oxalated plasma was rendered noncoagulable by treatment with Cγ alumina gel, and the filtrates incubated following the addition of calcium chloride for 24 hours. When thrombin was added fibrin was recovered in the expected amount (experiments 5 and 6 in Table 4). Incubation of this thrombin induced clot led to fibrinolysis.[2] However, the time for complete fibrin disappearance was longer in such specimens than in ones recalcified without alumina treatment because fibrinolysin was partly inactivated during the previous incubation period.

TABLE 5.

Fibrinolysis in hours in active plasma diluted with normal plasma.

	Undiluted plasma	Diluted to 50 p.c. with normal plasma	Diluted to 20 p.c. with normal plasma	Diluted to 10 p.c. with normal plasma
1	4	21		
2	4½	21		
3	21	21		
4	3	18*		
5	18	18*		
6	2¼	3		
7	4	21	21	∞
8	4	21	21	∞
9	3½	20	20	20
10	<18	18		
11	2½	3		
12	5	22	26	

* Plasma diluted with rabbit's plasma.

Although from these experiments it appears that only fibrin is attacked by the enzyme produced during electrically induced convulsions, it can be shown that a plasma possessing active fibrinolysin is able to break down additional amounts of fibrin produced by addition of normal plasma. The breakdown is not confined to homologous fibrin, since fibrin produced from rabbit's plasma was also attacked.

[2] The experiments have been carried out with human and commercial beef thrombin preparations; however, both occasionally contained fibrinolysin, whose absence was established prior to the experiment.

pH activity measurements carried out in veronal buffer (Michaelis) indicated dependence of pH optimum on the enzyme concentration. In an experiment in which lysis occurred in 2 hours optimal activity was found between pH 6·8–7·2, whilst in another case showing fibrinolytic activity after 9 hours, the optimal activity was between 6·6 and 7·0.

It should be pointed out that fibrinolysis could, in the majority of cases, be observed only in plasma diluted 25 times, whilst clots produced in whole blood did not show lysis when observed for much longer periods, indicating that the undiluted plasma contained inhibitors in amounts sufficient to prevent fibrinolysis. The presence of inhibitors could be demonstrated by comparing fibrinolysis in diluted plasma with that in the globulin fraction obtained by ammonium sulphate precipitation.

TABLE 6.

Comparison of fibrinolysis in diluted plasma and the isolated globulin fraction.

Experiment	Lysis in diluted plasma in hours		Lysis in globulin fraction in hours	
	Fibrin	Fibrinogen	Fibrin	Fibrinogen
1	19	no lysis in 20 hours	3	no lysis in 20 hours
2	9	no lysis in 20 hours	3	no lysis in 20 hours
3	19	no lysis in 20 hours	4½	no lysis in 20 hours
4	∞	no lysis in 20 hours	24	no lysis in 20 hours
5	∞	no lysis in 20 hours	∞	no lysis in 20 hours

The globulin fraction was isolated by addition of 2 ml. of 50 p.c. saturated ammonium sulphate solution to 1 ml. of oxalated plasma, (Christensen, 1946). The precipitate obtained after centrifugation was dissolved in 1 ml. of 0·15 M sodium chloride containing 0·01 M sodium oxalate.

Experiments 1, 2 and 3 are specimens of plasma after E.C.T., 4 and 5 are normal specimens.

It is apparent from these results that even in the absence of inhibitor the process is confined to the breakdown of fibrin, since fibrinogen was present after a prolonged incubation period.

The most probable explanation for the observed phenomenon is that the electric current induces activation of an inactive precursor of the fibrinolytic enzyme.

Investigation of the mechanism of proteolysis indicated that fibrin was certainly not broken down beyond the stage of polypeptides, because protein estimations carried out on trichloracetic acid precipitates, and also on fractions obtained by ammonium sulphate precipitation before and after incubation of specimens which showed fibrinolysis, did not give any significantly different results.

The peculiar behaviour of the fibrinolytic enzyme produced following E.C.T. made a comparison with other proteolytic enzymes desirable. A commercial trypsin preparation and fibrinolysin made by chloroform treatment of human serum according to Loomis, George and Rider (1947) were used.

The trypsin experiments were carried out in the case of plasmas 1–4 with 0·1 ml. of a 1 p.c. solution of trypsin, 0·1 ml. of oxalated plasma, 2·4 ml. of veronal buffer pH 7·9. In the case of plasma 5, 0·2 ml. of 1·5 p.c. trypsin solution was used.

TABLE 7.

Comparison between the action of trypsin and chloroform-fibrinolysin on human oxalated plasma.

Plasma specimen	Trypsin		Plasma specimen	Chloroform-fibrinolysin	
	Fibrin	Fibrinogen		Fibrin	Fibrinogen
1	200	<150	6	40	>40
2	200	<150	7	85	>140
3	250	130	8	64	89
4	260	180	9	72	89
5	<20	0·5			

Results indicate disappearance of substrates in minutes.

The fibrinolysin experiments were performed with 0·1 ml. of plasma, 1·0 ml. of the fibrinolysin preparation, 1·4 ml. veronal buffer. The pH of the reaction mixture was 6·7.

Experiments 6 and 7 were performed with a different fibrinolysin preparation to experiments 8 and 9.

Columns "Fibrin" indicate incubation at 37° C. with the addition of 0·1 ml. of 0·25 M calcium chloride. Columns "Fibrinogen" indicate incubation without calcium ions. Tests for the presence of fibrinogen were carried out with beef thrombin and calcium chloride.

From the results given in Table 7 it will be observed that trypsin attacked fibrinogen as well as fibrin; the former, however, was broken down at a faster rate than the latter. This phenomenon is in marked contrast to the E.C.T. induced fibrinolysis. Further, the fibrinolysin prepared by chloroform activation attacked fibrin at a slightly faster rate than fibrinogen.

The tryptic digestion of fibrin is also influenced by inhibitors present in plasma, since isolated fibrin is readily attacked by trypsin in a concentration which has no influence on fibrin produced by recalcification of whole plasma. From these results it is evident that the enzyme responsible for fibrinolysis in electrical shock is not identical with trypsin.

Convulsions were produced in four calves submitted to 3–8 shocks, each of a duration of 0·1 sec. at 180 volts. Fibrinolysis was not demonstrable in diluted plasma nor in the isolated globulin fraction. This negative result is unexpected since it has been reported by Loomis, George and Ryder (1947) that a globulin fraction from beef plasma can be converted by chloroform treatment into fibrinolysin, and Permin (1947) found that this activation can also be carried out by tissue fibrinokinase.

DISCUSSION.

The phenomenon of fibrinolysis, that is, the disappearance of a visible fibrin clot, in whole blood or plasma, has been observed by a number of workers in a variety of pathological and experimental conditions.

Tagnon *et al.* (1946) found fibrinolysis in shock due to severe haemorrhages or burns. This was observed in whole blood or moderately diluted plasma. The plasma of dogs subjected to severe bleeding likewise developed marked fibrinolytic properties.

Macfarlane and Biggs (1946) found fibrinolysis immediately after operation in 50 p.c. of patients, and Macfarlane, Biggs and Pilling (1947), on investigation

of this phenomenon, showed that states of anxiety, or injection of adrenaline, produced fibrinolysis. From their work it appears that any condition which would stimulate the sympathetic system would lead to fibrinolysis.

According to Delay and Soulairac (1943) many of the effects of electrical convulsions are like those produced by excitation of the sympathetic nervous system.

Fibrinolysis following adrenaline injection occurs in 24 hours or longer, when diluted plasma is tested. Plasma from patients submitted to E.C.T. showed fibrinolysis in times varying from 2 hours to 19 hours. In common with Macfarlane's observations, the E.C.T. induced fibrinolysis was of a transient nature.

Extensive studies on the protease of human serum by Christensen and MacLeod (1945) indicated that it is present as a precursor called plasminogen which can be activated, among other means, by streptokinase or by treatment with chloroform to the enzyme plasmin. The factor thus obtained differs from trypsin, having a pH optimum of 7·2, and attacking different linkages than trypsin in the protein molecule. Permin (1947) finds the pH optimum 6·0–6·5 for fibrinolysin produced by activation with tissue fibrinokinase. Further, Kaplan (1946) produced evidence that the enzyme obtained from serum by streptokinase activation is not identical with trypsin, because the kinases required for the respective enzyme activities are different. Tagnon *et al.* (1942) and Christensen (1945) observed that fibrinogen and other proteins, as well as fibrin, are attacked with equal velocity and therefore Christensen abandons the previous term fibrinolysin in favour of plasmin.

From the experiments reported here it is, however, quite apparent that plasma obtained shortly after electrically induced convulsions shows only fibrinolytic activity. No action on fibrinogen could be observed. It should be pointed out that the potency of Christensen's preparation was very much greater than was ever obtained in the plasma specimens following the electrical seizure. Although the proteolytic effect is partially inhibited by plasma components, the active fraction can be isolated by ammonium sulphate precipitation free of inhibitor, and yet the preferential breakdown of fibrin still persists. In many respects fibrinogen is more labile than fibrin. It is therefore interesting to note that plasmin in a low concentration should react with the more stable fibrin. Whether the discrepancy between the plasmin activities is due to the different mode of plasminogen activation, or to the difference of enzyme concentration, needs further investigation.

That the enzyme appearing following electrical shock in the plasma is different from trypsin could be shown quite clearly. The latter attacks fibrinogen in preference to fibrin, which is in agreement with observations made by Ferguson *et al.* (1947).

It is very likely that fibrinolysis following electrically induced shock is due to the same stimulus as observed by Macfarlane and co-workers, although no

direct evidence is available at the moment that adrenaline is the factor eliciting plasminogen activation. The breakdown of fibrin by the plasmin concentration obtainable in E.C.T. does not go beyond the polypeptide stage, since no difference in trichloracetic acid precipitable protein could be observed in specimens before and after incubation. This is in line with observations made by Garner and Tillet (1934), who found that the breakdown of fibrin by fibrinolysin produced by streptokinase activation was not very profound, as the degradation products were precipitated by ammonium sulphate, a fact confirmed by Holmberg (1944). Likewise Seegers *et al.* (1945) found that fibrinogen isolated from bovine plasma loses its coagulability when kept under sterile conditions at room temperature, but the breakdown products show, apart from the non-coagulability by thrombin, little difference in other respects from native fibrinogen.

Tagnon *et al.* (1946) reported that severe haemorrhagic shock led, in addition to fibrinolysis, to reduction of prothrombin activity. No influence on the prothrombin level could be observed under our experimental conditions.

It would appear that the low concentration of plasmin produced by sympathetic stimulation is of physiological significance, and this process may serve to remove fibrin when intravascular coagulation takes place, without interfering with the haemostatic mechanism.

SUMMARY.

Following convulsions induced by alternating current in humans, changes in the composition of blood are a slight haemo-concentration, a moderate increase in plasma proteins, and a transient fibrinolytic activity. The latter is confined to breakdown of fibrin, fibrinogen not being attacked. This phenomenon is in contrast to tryptic action, and is additional evidence that the plasma protease is different from trypsin.

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THE METABOLISM OF ERYTHROCYTES

II. THE DECARBOXYLATION OF OXALACETATE BY BLOOD.

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In recent years the metabolism of oxalacetic acid has acquired ever-increasing importance. Its rôle in the combustion of carbohydrates and fats has been very thoroughly investigated (Krebs, 1943; Breusch, 1943; Buchanan *et al.*, 1945; Lehninger, 1945; Weinhouse *et al.*, 1946, etc.).

Furthermore, it is undoubtedly the key substance in the heterotrophic fixation of CO_2 by animals (Evans *et al.*, 1943), plants (Gollub and Vennesland, 1947) and bacteria (Krampitz and Werkman, 1941; Krampitz, Wood and Werkman, 1943). Surprisingly enough, no definite enzyme which specifically affected oxalacetate was described prior to 1941, but Breusch (1939) reported a heat-stable factor in various animal tissues which caused decarboxylation of oxalacetate. The first actual enzyme described (Krampitz and Werkman, 1941) was found in certain bacteria; it was a magnesium or manganese protein complex independent of co-carboxylase and partly thermolabile. A very similar system was reported shortly afterwards for pigeon liver (Evans, Vennesland and Slotin, 1943) which differed from the bacterial enzyme in one important aspect, viz. that manganese only was active in conjunction with the enzyme, magnesium failing to reactivate dialysed preparations. This enzyme was completely inhibited by 10^{-2}M malonate. The products of both the above processes were shown to be CO_2 and pyruvate, the reaction in each case proceeding almost to completion.

An apparently entirely different process was subsequently reported from the same laboratory (Vennesland and Evans, 1944) to occur in pig's heart; an *oxidative* decompositon, in which the disappearance of a molecule of oxalacetate was accompanied by the uptake of an atom of oxygen and the evolution of a molecule of CO_2, was described. The product of the decomposition was shown to be malonic acid, and the system again required manganese. In a later communication (Vennesland, Evans and Francis, 1946) the enzyme was claimed to be identical with metmyoglobin, which, when prepared according to standard methods, showed exactly the same effect in the presence of Mn^{++}. During the

[1] This work was supported by an apparatus grant from the National Health and Medical Research Council.

decomposition of the substrate, partial destruction of the enzyme occurred. The authors liken the process especially to the oxidative decomposition of dioxymaleic acid by cytochrome *c* in the presence of Mn^{++}.

When the qualitative discovery of a decarboxylation of oxalacetate by washed rabbit erythrocytes was first reported (Nossal, 1948a) it was thought that one or other of the previously described enzymes might be in operation. Subsequent more thorough quantitative examination of the problem showed, however, that either a much more complex, or else an entirely different, mechanism appeared to occur throughout rabbit's blood. Since boiling at pH 6·5 seemed to cause partial destruction of activity, whereas boiling at pH 3·5–4·0 not only failed to reduce, but actually *increased*, the speed of decarboxylation, the existence of *two* mechanisms was suggested (Nossal, 1948b). One was thermolabile, apparently enzymatic, affected by Mn^{++} (but less markedly than previously described enzymes) and operative at roughly physiological pH values. The other came into operation at low pH values, and appeared to be due to the liberation of some substance under those conditions; this liberation was *accelerated* by boiling at pH 3·5–4·0.

The present work represents further investigations directed towards the identification of the mechanism responsible for oxalacetate decarboxylation by blood. Results of the preliminary report (Nossal, 1948b) are included in greater detail here.

A study of the effects of various ions on the autodecomposition of oxalacetate is also included.

METHODS.

Materials.

The material used throughout was rabbit's blood. The blood was defibrinated by shaking with glass beads during withdrawal of each sample. The erythrocytes were separated by centrifugation at 3,000–4,000 r.p.m. for 10 minutes, and the clear plasma removed by careful suction. In the case of comparative experiments, cell suspensions, plasma samples and erythrocyte haemolysates were prepared in such a way that all were of concentrations equal to their original concentrations in whole blood. Otherwise, haemolysates were generally prepared by treatment of the separated cells with two volumes of distilled water.

Acetone powder extracts were made by treatment of the material under examination (whole blood, erythrocytes, plasma or haemolysate) with five volumes of ice-cold acetone, removal of the precipitate by filtration, drying of the precipitate *in vacuo* at 0° C., and extraction of the powder thus obtained with 8–10 volumes of water or buffer at 38° C. for 10–20 minutes.

Dialysis.

The material to be dialysed was placed in standard dialysis-tape bags, and these were submerged in distilled water. Several procedures were tried: (a) shaking at room temperatures for up to 20 hours; (b) standing at 0° C. for 40 hours; (c) shaking at 5° C. for up to 40 hours. The control samples were subsequently diluted to the same extent as the dialysed samples.

Oxalacetic Acid.

The samples used in these experiments were generous gifts from Professor J. G. Wood and Mr. H. R. Marston. It was soon found that even neutral solutions of oxalacetate underwent rapid autodecomposition. At pH 8, this process is greatly retarded. However, the use of such solutions of substrate causes the introduction of an unknown factor into the final results, viz., the CO_2 dissolved in the 0·2 ml. of substrate solution and liberated when tipped into an acid buffer. Therefore, the following procedures were adopted: when it was desirable to use the same substrate sample for several experiments, and when the preparations were so active as to render the error referred to above negligible, the pH of the substrate was adjusted to approximately 8. In all other cases, the substrate solution was prepared immediately prior to the commencement of the experiment, and almost neutralized. In both cases, the concentration of substrate solutions was such that when 0·2 ml. was diluted to 3·0 ml. the final concentration of oxalacetate was 10^{-2}M or 5×10^{-3}M.

Buffers.

Initially, ordinary phosphate-saline was used for the whole cell experiments (2×10^{-3}M Na_2HPO_4 and KH_2PO_4 in 0·9 p.c. NaCl). When the great importance of carefully controlling the pH became apparent, comparative experiments with phosphate buffer and 2M acetic acid—sodium acetate buffer were carried out. Since no great differences in the decarboxylation in the presence of the two buffer systems were observed, all subsequent experiments were carried out with the more powerful acetate buffer.

Measurement of Decarboxylase Activity.

Estimation of CO_2 due to substrate decomposition was carried out in standard Warburg equipment by the direct CO_2 technique. Blank CO_2 outputs and oxygen uptakes of the haemolysates were generally so small when compared with the rate of decarboxylation of oxalacetate, that it was not necessary to carry out these measurements in the majority of cases. CO_2 *evolution* only was measured and at pH values from 6·0–7·0 correction for dissolved CO_2 was made according to Johnson (quoted in Umbreit *et al.*, 1945). The experiments were carried out at 37–38° C. The Warburg bath was of the type previously described (Nossal, 1948c) and temperatures could be kept constant to within 0·02° C. for the experimental period.

Readings were taken at various intervals (and continued for differing periods of time after tipping in the substrate) in accordance with the activity of the system under examination.

RESULTS.

Autodecomposition of Oxalacetate.

In a previous communication (Nossal, 1948b) it was reported that boiling of a diluted haemolysate at low pH caused *increases* in the rate of decarboxylation of oxalacetate, when the activities of boiled and unboiled systems were compared. It was therefore thought advisable to investigate the effect of various substances (which might conceivably be formed when cellular material is boiled at pH 3·5–5·0) on the autodecomposition of the substrate.

Firstly, the very great variation of this process with pH must be mentioned. Breusch (1939) reported that the decomposition of oxalacetate was more rapid in acid solution than at neutrality, and less rapid in alkaline solution. Fig. 1 gives a more precise picture of the effect of pH variation on oxalacetate decomposition.

The very great increase during the transition from pH 7 to pH 3·5 can clearly be seen. It must be noted here that all subsequent results are *corrected* for this autodecomposition by

the inclusion of the appropriate buffer controls into each experimental run, unless separate values for autodecomposition are quoted.

Since aniline citrate quantitatively decarboxylates oxalacetate (Edson, 1935) it was thought that ammonia, which could well be liberated by boiling at acid pH, might have a similar action. However, concentrations of NH_3 from 10^{-3}M to 10^{-2}M at pH 6·5 had absolutely no effect on the process, so that this substance may be eliminated as featuring in the process under examination.

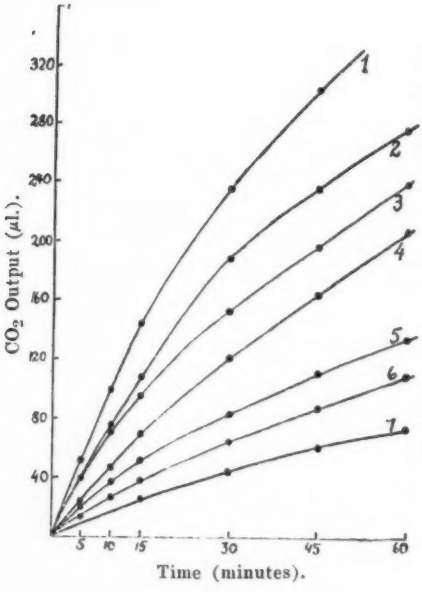

Fig. 1.

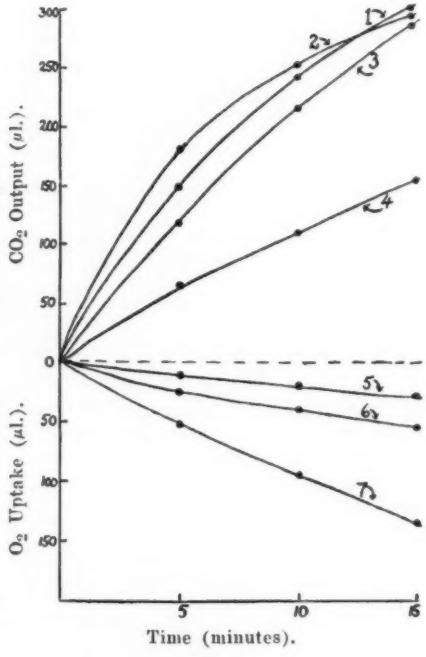

Fig. 2.

Fig. 1. Effect of pH variation on oxalacetate autodecomposition.

Conditions: all cups contained 2·8 ml. buffer and 0·2 ml. substrate (S.B.). (Final concentration 10^{-2}M). T = 38° C. Curve 1, pH 3·5; Curve 2, pH 4·0; Curve 3, pH 5·0; Curve 4, pH 5·5; Curve 5, pH 6·0; Curve 6, pH 6·5; Curve 7, pH 7·0.

Fig. 2. Effect of ferrous and ferric iron on oxalacetate autodecomposition.

Conditions: pH: 5·0, oxalacetate in one side-arm, iron in the other. CO_2 outputs are all corrected for autodecomposition and O_2 uptakes. Curve 1, 10^{-3}M Fe++; Curve 2, 10^{-2}M Fe++; Curve 3, 10^{-3}M Fe+++; Curve 4, 10^{-2}M Fe+++; Curve 5, blank O_2 uptake of 10^{-3}M Fe++; Curve 6, blank O_2 uptake of 10^{-2}M Fe++; Curve 7, O_2 uptake of 10^{-2}M Fe++ in presence of oxalacetate.

Iron was also considered a possibility, since it might be split off from haemoglobin under the above-mentioned conditions.

It was previously reported (Nossal, 1948b) that ferric iron was slightly more active than

ferrous iron. This claim was based on an experiment in which the iron was placed into the centre of each cup with the acetate buffer. Under the experimental conditions, a steady oxygen uptake due to the oxidation of Fe++ to Fe+++ was observed, and the decarboxylation of oxalacetate corrected accordingly. Even then, ferric iron was more active than ferrous (Table 1).

TABLE 1.

Effect of iron on oxalacetate autodecomposition.

All cups contain 2M acetate buffer, pH 6·5. Iron in main compartment. Oxalacetate in side-bulb such that final concentration is 10^{-2}M. Final volume: 3·0 ml. Temperature: 37° C. Duration: ½ hour.

Agent.	Concentration (M).	O_2 uptake of Fe++ only (μl.).	Increases in CO_2 output (after correction for blank) (μl.).
Fe++	10^{-2}	136	—
Fe++	10^{-2}	—	100 (not corrected for O_2 uptake)
Fe++	10^{-2}	—	256 (corrected for O_2 uptake)
Fe++	10^{-3}	negligible	242
Fe+++	10^{-2}	0	324
Fe+++	10^{-3}	0	294

The observation that in those cups which contained oxalacetate + 10^{-2}M Fe++, a deeper brown colour resulted than in those which contained Fe++ and buffer only, led to the discovery that during the decarboxylation of oxalacetate by Fe++ a marked increase in oxygen uptake occurs. A typical experiment is shown in Fig. 2. It is seen that under these conditions Fe++, when duly corrected for O_2 uptake, is considerably *more* active than Fe+++. Dilution of Fe++ from 10^{-2}M to 10^{-3}M causes virtually no loss of activity. The rate of O_2 uptake in the presence of 10^{-2}M Fe++ and oxalacetate is such as to suggest that for every molecule of CO_2 given out, one atom of O_2 is taken up. Whether Fe++ causes an *oxidative* decarboxylation of oxalacetate, or whether oxalacetate merely causes oxidation of Fe++ to Fe+++, cannot as yet be said.

The most remarkable aspect of iron catalysis is the effect of dilution on the action of iron at various pH values. It can be seen from Table 2 that below pH 6·5 10^{-3}M Fe+++ is much *more* active than 10^{-2}M Fe+++, whereas at and above that value, the behaviour is normal. Since it is in the vicinity of pH 6·5 that oxalacetate itself changes colour (probably due to enolization), and since the behaviour of ferric ions in acetate is complex, there seems to be no simple explanation of this effect. No changes in O_2 uptake and/or blank CO_2 output with varying pH were observed, which could account for the inversion of activity.

The effect of iron is completely nullified by the addition of a strong solution of cupferron. This is of considerable importance, as will be seen later.

The next substance to be investigated was copper which exists in the blood to the extent of 2 μ gm./ml. Since the autodecomposition was sensitive to 10^{-3}M Fe++/Fe+++, a certain effect by copper was not unexpected. Table 3, however, shows that at low concentrations copper has an even more marked effect than iron.

TABLE 2.

Effect of dilution on the activity of ferric iron at varying pH values.

Conditions: 10^{-2}M oxalacetate, total vol.: 3·0 ml., iron in centre.

Exp.	pH.	Molarity of Fe+++.	CO_2 outputs (µl) for the following intervals after tipping (minutes):			
			5	10	15	30
1	5·0	—	28	56	83	—
1	5·0	10^{-2}	66	120	158	—
1	5·0	10^{-3}	151	262	305	—
1	6·0	—	13	27	43	78
1	6·0	10^{-2}	33	69	90	124
1	6·0	10^{-3}	56	110	147	217
1	7·0	—	3	7	10	27
1	7·0	10^{-2}	27	59	87	138
1	7·0	10^{-3}	7	18	30	58
2	6·0	—	26	—	50	97
2	6·0	10^{-2}	78	—	114	153
2	6·0	10^{-3}	95	—	162	250
2	6·5	—	32	—	47	80
2	6·5	10^{-2}	73	—	118	174
2	6·5	10^{-3}	35	—	79	121

TABLE 3.

Effect of copper on oxalacetate autodecomposition.

Conditions: as for Table 1. Duration: 30 minutes.

Molarity of Cu++ (final).	Increases in CO_2 evolution (µl.).
10^{-3}	329
10^{-4}	183
10^{-5}	30
10^{-6}	12

It was, therefore, not unreasonable to assume that both Fe++/Fe+++ and Cu++ could be concerned in the "unphysiological" decarboxylation which was observed after boiling the various preparations at low pH values. As in the case of iron, the effect of copper was completely abolished in the presence of appropriate amounts of cupferron. This will again be discussed later. Cupferron alone had no influence on the autodecarboxylation rate, so that the latter, under the experimental conditions employed, is *not* due to impurities of copper and/or iron.

After both Fe and Cu had been found active, a number of other metallic ions were investigated. These were: Mn++, Mg++, Ni++, Al+++, Co++, Ca++, Pb++, Hg++, and Sn++++.

The effects of these metals are shown in Fig. 3, from which it can be seen that some act almost as rapidly as aniline citrate. They are, therefore, suitable for simple, quantitative assays of oxalacetate under certain conditions.

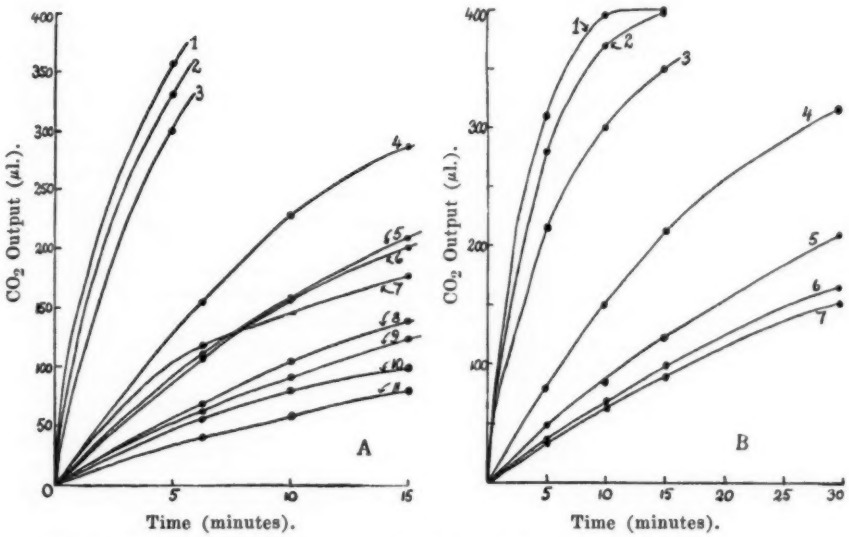

Fig. 3. Effect of metallic ions on oxalacetate autodecomposition.
Conditions: pH: 5·0, metals in main compartment, oxalacetate: 10^{-2}M.
For Part A: metal concentration = 10^{-2}M.
For Part B: metal concentration = 10^{-3}M.

Part A: Curve 1, Cu++; Curve 2, Al+++; Curve 3, Ni++; Curve 4, Co++; Curve 5, Mn++; Curve 6, Pb++; Curve 7, Fe+++; Curve 8, Mg++; Curve 9, Sn++++; Curve 10, Blank and Hg++; Curve 11, Ca++.

Part B: Curve 1, Cu++; Curve 2, Al+++; Curve 3, Fe+++; Curve 4, Ni++; Curve 5, Co++; Curve 6, Mg++, Mn++; Curve 7, Blank.

The considerable variations in activity are seen at both final concentrations. At 10^{-2}M, the differences between the three most active metals, Cu++, Al+++ and Ni++, are ill-defined, but those between the less active metals are readily seen. On the other hand, at 10^{-3}M the less active metals nearly all converge (and, therefore, only a few are shown in the Figure), whereas Cu++, Al+++ and Ni++ are readily differentiated. The striking effect of the dilution of Fe+++ is again seen. It is of interest to note that mercuric ions were quite inactive and that calcium actually caused a slight retardation of the autodecomposition.

Specificity.

As reported previously (Nossal, 1948a and 1948b), the systems here described seem to show considerable specificity. No decarboxylation of α-ketoglutarate, pyruvate or malate was observed in any experiments.

Distribution.

Decarboxylative activity has been observed in erythrocytes, erythrocyte haemolysates, plasma, whole blood and various acetone powder extracts.

When measured as *total* evolution of CO_2 over two-hour periods at pH 6·5, the various

parts of blood examined showed roughly equal activity. This is somewhat deceptive, as *rate* experiments show that the haemolysate causes the most rapid initial decarboxylation. The fact that whole blood is no more active than erythrocytes or plasma would indicate that there is the same mechanism distributed in both these fractions. If this were not so, one might expect the activity of whole blood to equal, at least approximately, the sum of the activities of plasma and erythrocytes.

It was noticed that the plasma was never colourless. Spectroscopic examination of some of the samples often showed the two characteristic absorption bands of oxyhaemoglobin. On our data, however, it cannot as yet be definitely stated, whether the process in plasma represents a contamination from the erythrocytes, or whether plasma itself contains the same, or a similar, or another, decarboxylation mechanism.

Tested at pH 6·5, extracts from plasma were more active than those from whole blood. etc. Typical distribution experiments are cited in Table 4. It must be remembered that the activities of blood constituents and the various APX's (acetone powder extracts) are not quantitatively comparable for obvious reasons, but that each member of each group is comparable.

TABLE 4.

Distribution of decarboxylase activity in blood and acetone powder extracts.

Fillings: all cups contain 2·0 ml. of the catalyst solution adjusted to pH 6·5, 10–2M oxalacetate, and buffer (2M acetate) pH 6·5. Final volume: 3·0 ml. Duration: 2 hours. CO_2 outputs are duly corrected for blank CO_2 formation and also for oxygen uptake, as well as oxalacetate autodecomposition.

System.	CO_2 formed from oxalacetate (μl.).
Whole blood	124–137
Erythrocytes	100–154
Haemolysate	147–169
Plasma	128–151
Acetone powder extract:	
Whole blood	53–107
Haemolysate	48– 95
Erythrocytes	61–115
Plasma	98–174
Residue of above APX re-extracted.	67–100

It is of interest to note that in spite of varying periods of extraction and the use of different extracting agents, the residue obtained by spinning down the APX invariably retained at least some activity. The activities of different APX's differed rather more than those of different blood samples.

Effects of Cofactors on the Process.

The bacterial and animal enzymes previously described depended on Mg++ and/or Mn++ for full activity. Thiamine and cocarboxylase apparently did not function in the systems.

Thiamine in the experiments described here had no effect whatever on any of the systems tested. In a number of experiments, washed, intact erythrocytes were incubated, (a) alone, (b) with thiamine, and (c) with thiamine plus glucose for one hour, and the decarboxylase activity subsequently investigated. Since the erythrocytes are known to possess powerful phosphorylating mechanisms, it was not unreasonable to expect that, especially in (c),

thiamine pyrophosphate would be formed if this substance were a cofactor of the decarboxylase (since then surely the cell would have to have a mechanism for thiamine pyrophosphate synthesis). However, no difference in the rates of decarboxylation by systems (a), (b) and (c) was observed. This must mean either that the cofactor is not involved in the process under test, or else that, under the experimental conditions, the enzymes always contain optimal, or more than optimal, amounts of cocarboxylase. Especially in view of the findings of previous workers on oxalacetate decarboxylation, the first explanation seems more likely to be correct.

In view of the finding of Speck *et al.* (1946), that normal erythrocytes accumulate citrate in the presence of pyruvate and oxalacetate, the effect of pyruvate on the decarboxylation was tested. If the observed CO_2 evolution were due to a partial combustion of pyruvate by means of the tricarboxylic acid cycle, then pyruvate should increase the CO_2 output in the presence of oxalacetate. Since addition of 10^{-2}M lithium pyruvate had no effect whatever on the rate of CO_2 formation, or the total CO_2 formed, it was concluded that such a mechanism does not account for the CO_2 formation described here.

Magnesium and manganese tested against the undialysed systems gave very interesting results. No increases whatever were observed with whole blood, plasma, etc., for concentrations up to 10^{-2}M of the ions. At those concentrations, the ions definitely accelerate the autodecomposition of the substrate. It, therefore, seems as though removal of ionic Mg^{++} and Mn^{++} occurs in the blood at pH 6·5. It must be mentioned that a certain amount of metal phosphate appeared to be formed in the controls which contained phosphate buffer.

TABLE 5.

Effect of dilution on decarboxylase activity.

Conditions: 10^{-2}M oxalacetate, total volume: 3·0 ml., T = 38° C., experimental period as indicated.

System.	pH.	Dilution.	Duration (min.).	Total CO_2 evolved (corrected for autodecomposition) (μl.).
Haemolysate	6·5	none	60	159
Haemolysate	6·5	1 in 2	60	140
Haemolysate	6·5	1 in 5	60	112
Haemolysate	6·5	1 in 10	60	76
Haemolysate	5·0	1 in 2	30	157
Haemolysate	5·0	1 in 4	30	100
Haemolysate	5·0	1 in 10	30	43
Haemolysate	5·0	1 in 20	30	19
Haemolysate	4·0	none	30	119
Haemolysate	4·0	1 in 2	30	113
Haemolysate	4·0	1 in 3	30	109
Haemolysate	4·0	1 in 5	30	84
Haemolysate	4·0	1 in 10	30	60
Acetone powder extract	6·5	none	60	105
Acetone powder extract	6·5	1 in 10	60	34
Acetone powder extract	6·5	none	30	66
Acetone powder extract	6·5	1 in 5	30	31

(Several previous workers, however, have carried out all their experiments in this buffer.) Occasionally, slight increases in activity with APX's of low decarboxylase power were observed by high concentrations of both Mn++ and Mg++. The effects of these ions on dialysed systems will be dealt with later. The results here quoted suggest that all the systems tested against Mn++ and Mg++ contained sufficient amounts of either ion (or both ions) for optimal activity, so that further additions were ineffective.

As will be seen later, both suggestions (i.e. nonparticipance of cocarboxylase and independence or saturation of the systems with metallic ions) are supported by the results of dialysis experiments.

Effect of Dilution.

Table 5 shows the effect of dilution on the total CO_2 evolution by various systems over 30 minutes. The experiments were carried out by diluting the catalyst and maintaining the substrate at constant level throughout.

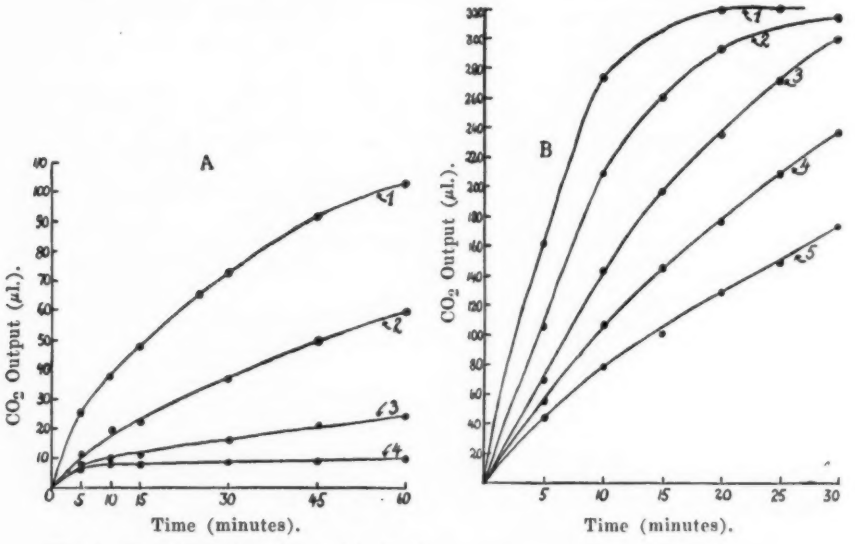

Fig. 4. Variation of activity with dilution.
Fillings: 2·0 ml. catalyst, 0·8 ml. buffer, 10–2M oxalacetate. Total vol.: 3·0 ml.

Part A: activity of a haemolysate. (*Note*: all readings are corrected for autodecomposition) pH: 6·5. Curve 1, Diluted 1 in 2; Curve 2, 1 in 4; Curve 3, 1 in 10; Curve 4, 1 in 20.

Part B: activity of a haemolysate at pH 3·5. (*Note*: autodecomposition shown on a separate curve, other curves not corrected for it.) Curve 1, undiluted; Curve 2, diluted 1 in 2; Curve 3, diluted 1 in 5; Curve 4; diluted 1 in 10; Curve 5, autodecomposition (buffer only).

Actually it is more correct to compare the activities of undiluted and diluted samples graphically, as, in some cases, the diluted systems begin to "catch up" on the stronger samples; Fig. 4 shows two different effects of dilution.

It was considered of interest to examine the effect of Mg++ and Mn++ on diluted systems, to see whether the decrease in activity was due to a fall of the activator concentration

to sub-optimal levels. On numerous samples of varying dilution (up to 1:20), concentrations of Mn++ and Mg++ of up to 10^{-2}M had no significant effects after due correction had been made for the increases in autodecomposition caused by the metals. This constitutes a further argument in favour of the view that these metals may not be components of the decarboxylation system.

Effect of Dialysis.

A large number of samples was dialysed against distilled water for varying periods, to see whether soluble, diffusible co-factors and/or activators featured in the decarboxylation.

The decreases in activity after dialysis were invariably small. Samples dialysed unbuffered were adjusted to various pH values after dialysis to see whether the process was affected more at one pH value than at any other. However, the differences between dialysed and control samples for pH values from 6·5 to 3·5 were all of the same order. Two typical examples are shown in Fig. 5.

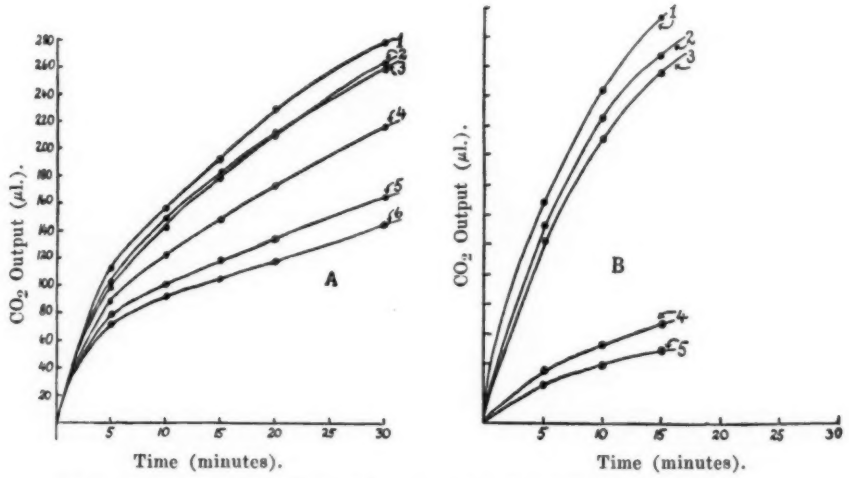

Fig. 5. Dialysis and the effect of Mn++ on decarboxylation rate.
Fillings: as in Fig. 4, Mn++: 10^{-3}M.

Part A: plasma sample, pH 6·0. Curve 1, undialysed plasma + Mn++; Curve 2, dialysed plasma + Mn++; Curve 3, undialysed plasma; Curve 4, dialysed plasma; Curve 5, buffer + Mn++; Curve 6, buffer only.

Part B: haemolysate pH 6. Curve 1, dialysed sample + Mn++; Curve 2, undialysed sample; Curve 3, dialysed sample; Curve 4, buffer + Mn++; Curve 5, buffer only.

It will be seen that the plasma inactivation is less than 20 p.c., and that of the haemolysate even less. Furthermore, although Mn++ increases the control plasma activity only to the same extent as the buffer, a somewhat more marked increase is observed in the case of the dialysed plasma sample, which is thereby restored to the level of the control. The haemolysate samples respond roughly to the same extent to Mn++. Magnesium in all cases was even less active than manganese.

It is of interest to mention here that frequently Mn++ caused smaller increases in the activity of haemolysates than in buffer alone. It appears, therefore, as though removal of the metal (e.g. as phosphate) may occur. This would explain the lowered effect. After dialysis, phosphate concentration would be lower, and thus the Mn++ could exert its full effect.

The effects of both dialysis and Mn++, as a whole, are so slight as to be inconclusive. On the other hand, it is quite obvious that the system in blood is, in this respect, unlike the decarboxylation in pigeon liver or bacteria, where Mn++ and/or Mg++ exerted really pronounced effects on the activities of metal-deficient preparations.

The only conclusion other than that metals do not feature in the decarboxylation, would be to assume that the metal is so firmly attached to the protein that it cannot be removed by dialysis.

It seems also certain that the blood decarboxylation resembles those previously described in being independent of cocarboxylase.

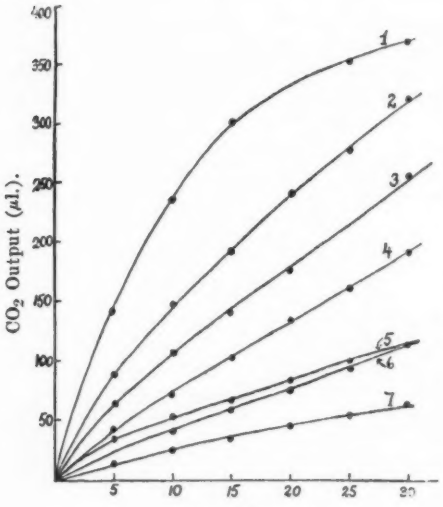

Fig. 6: Effect of pH variation on the decarboxylation activity of plasma.

Conditions: as in Fig. 4.

Curve 1, plasma pH 3·5; Curve 2, plasma pH 4·5; Curve 3, buffer pH 3·5; Curve 4, plasma pH 5·5 and buffer pH 4·5; Curve 5, buffer pH 5·5; Curve 6, plasma pH 6·5; Curve 7, buffer pH 6·5.

Variation of Activity with pH.

The behaviour of the decarboxylation at various pH values is undoubtedly one of the most interesting features of the process.

Initially, it was thought that a complete range of activities from pH 9–pH 4 could be obtained. At alkaline pH values, the evolved, initial dissolved and final dissolved CO_2 was to be measured, whereas below pH 6·5, measurement of CO_2 evolution would be sufficient. It was soon found, however, that the initial and final dissolved CO_2 volumes were too large to measure in the Warburg equipment employed. Furthermore, additional complications were introduced by the fact that acid liberates appreciable amounts of O_2 from oxyhaemoglobin.

Measurements above pH 7·0 were, therefore, not made. At any rate, it was found that decarboxylation activity increased during the transition from pH 7·0 to pH 6·0, so that the optimum value very probably lay on the acid side of pH 6·0.

Samples were accordingly tested at pH values between the range of 3·5 and 7·0. This applies to haemolysates, plasma, and acetone powder extracts. As can be seen from the accompanying results (Fig. 6, Table 6), maximal activity for all samples was observed at the lowest pH value tested. Considerable care must be taken in the comparison of results: If the total CO_2 evolved over a given period (e.g. 30 minutes) is taken as an indication of activity, deceptive results are obtained (see Nossal, 1948b, in connection with plasma), as the most active samples have slowed down, and their blanks are catching up with them, so that the final difference between experiment and blank can seem less for a more active specimen than

for another of actually smaller activity (See Fig. 6). It must be noted that different plasma and APX samples particularly differed widely in activity, but that for each individual sample activity varied with pH in a typical manner.

Table 6 shows typical experiments with haemolysate and plasma. It can be seen that in both cases considerable increases in activity are observed as the pH is lowered. Spectroscopic examination showed typical oxyhaemoglobin spectra at pH 6·5, a mixture of the latter with methaemoglobin at pH 5, and mainly acid haematin at pH 3·5. This finding was supported by preliminary spectrophotometric examination of the samples in a Coleman Universal spectrophotometer.

TABLE 6.

Decarboxylation activity at various pH values.

Conditions: 10^{-2}M oxalacetate, total volume = 3·0 ml. per cup. T = 38° C. Blanks contain buffer and substrate only.

System.	pH.	µl. CO_2 evolved for the intervals after tipping (in minutes) indicated by the figures in brackets:			
Buffer	7·0	38 (5)	53 (10)	61 (15)	
Haemolysate	7·0	102 (5)	153 (10)	192 (15)	
Buffer	6·0	68 (5)	83 (10)	97 (15)	
Haemolysate	6·0	178 (5)	268 (10)	334 (15)	
Buffer	4·0	96 (5)	136 (10)	183 (15)	
Haemolysate	4·0	356 (5)	443 (10)	453 (15)	
Buffer	3·5	98 (5)	139 (10)	189 (15)	
Haemolysate	3·5	384 (5)	472 (10)	495 (15)	
Acetone powder extract	5·5	13 (5)	20 (10)	20 (15)	corrected for autodecomposition
Acetone powder extract	4·5	12 (5)	22 (10)	26 (15)	
Acetone powder extract	3·5	28 (5)	41 (10)	41 (10)	
Plasma	6·5	19 (5)	21 (10)	28 (15) 41 (30)	corrected for autodecomposition
Plasma	5·5	43 (5)	44 (10)	57 (15) 70 (30)	
Plasma	4·5	53 (5)	65 (10)	74 (15) 86 (30)	

Up to date, the process has not been examined at pH values lower than 3·5, for the following reasons:

Although acetate buffer is by no means optimal at pH 3·5, determination of pH after completion of the experiment showed that, under the conditions employed, the pH had never risen by more than 0·1. Below pH 3·5, however, more significant changes were observed, due to the liberation of base accompanying oxalacetate decomposition. It was thought undesirable to introduce an additional unknown factor by the use of entirely different buffers for the very low pH values.

For the time being, therefore, the main conclusion from the work over the pH range is that erythrocytes contain one or more decarboxylation systems, the total effect of which is very much greater at pH 3·5 than at physiological pH values.

The Effect of Boiling at Varying pH Values.

It was previously reported (Nossal, 1948b) that pH had a considerable bearing on the subsequent activity of a boiled sample. If a haemolysate (or an acetone powder extract) is diluted without pH adjustment, and then boiled, the activity of the boiled sample is either

almost zero, or else very much less than the correspondingly diluted, unboiled control sample at the same pH. This finding was taken as at least one argument for the claim that at pH 6·5 the decarboxylation was heat-labile and therefore probably enzymatic (Table 7).

However, when it was observed that the pH of the samples at the time of boiling had a very profound effect on the subsequent activity, it was realized that the stipulation of a simple, enzymatic catalysis would have to be modified. No sample adjusted to pH 6·0 or less ever showed a complete lack of activity after being boiled. Samples above pH 5·0 occasionally exhibited a slightly diminished activity, but below that pH boiling caused either no significant change, or else increases in activity were observed. Typical examples are shown in Table 7.

TABLE 7.

The effect of boiling under varying conditions on decarboxylation activity.

T = 38° C., oxalacetate concentration = 10^{-2}M, total volume/cup = 3·0 ml.

System.*	pH.	Boiled (B) or unboiled (UB).	CO_2 evolution (μl.) corrected for autodecomposition: (figures in brackets represent the time in minutes, after tipping at which measurements were made).				
APX	6·5	UB	18 (5)	56 (30)	82 (60)	103 (120)	
APX	6·5	B	2 (5)	14 (30)	20 (60)	24 (120)	
Haem. (1:10)	6·5	UB	—	—	—	78 (120)	
Haem. (1:10)	6·5	B	—	—	—	0 (120)	
Haem. (less diluted)	6·5	UB	53 (5)	96 (15)	127 (30)	151 (60)	
Haem. (less diluted)	6·5	B	21 (5)	26 (15)	30 (30)	35 (60)	
Plasma	6·0	UB	15 (5)	22 (10)	28 (15)	41 (30)	
Plasma	6·0	B	17 (5)	25 (10)	33 (15)	60 (30)	
Plasma	4·0	UB	78 (5)	101 (10)	124 (15)	—	
Plasma	4·0	B	105 (5)	138 (10)	151 (15)	—	
Plasma	3·5	UB	88 (5)	206 (15)	261 (25)	—	uncorrected for autodecomposition
Plasma	3·5	B	143 (5)	290 (15)	344 (25)	—	
Haem.	6·0	UB	53 (5)	107 (10)	144 (15)	199 (30)	
Haem.	6·0	B	49 (5)	98 (10)	126 (15)	170 (30)	
Haem.	3·5	UB	46 (5)	86 (10)	112 (15)	141 (30)	
Haem.	3·5	B	59 (5)	121 (10)	154 (15)	176 (30)	

* APX = acetone powder extract; Haem. = haemolysate; brackets show the pairs chosen for comparison.

The heat inactivation shown under certain conditions thus obviously depends on the pH. To see whether the inactivation was reversible, certain samples were boiled prior to pH adjustment (when deactivation should occur) and the pH was then adjusted to a value at which no decrease in activity was known to occur on boiling (e.g. pH 3·5 to 5·0). If the deactivation were irreversible, then little, if any, activity should be found in the samples thus treated. That this was not the case can be seen from Table 8.

Therefore, the conclusions to be drawn from the boiling experiments are the following: Boiling at alkaline pH leads to the formation of less active systems, whereas at fairly acid pH no change, or even an increase in activity is observed. The deactivated systems show very significant activity when examined at lower pH values. It is thus probable that the less active systems (formed at alkaline pH values) when suspended at low pH values are converted to similar (or identical) systems as those formed by boiling at acid pH.

TABLE 8.

Effect of boiling before and after pH adjustment in decarboxylation activity.

Conditions: as in Table 7.

System.	Comments.	Final. pH.	μl. CO_2 evolved (corrected for autodecomposition) over the periods (in minutes) indicated by the figures in brackets.		
Haem.	Unboiled	3·5	178 (5)	230 (10)	235 (15)
Haem.	Boiled prior to pH adjustment	3·5	165 (5)	213 (10)	229 (15)
Haem.	Boiled after pH adjustment	3·5	167 (5)	233 (10)	239 (15)
Plasma	Unboiled	4·0	80 (5)	105 (10)	130 (15)
Plasma	Boiled prior to pH adjustment	4·0	107 (5)	142 (10)	157 (15)
Plasma	Boiled after pH adjustment	4·0	83 (5)	116 (10)	136 (15)

The boiled samples were next divided into precipitate and supernatant to determine in which fraction the activity was to be found. (It should be remembered here that the full activity could never be extracted by water from blood acetone powders.)

A haemolysate was accordingly adjusted to pH 6·5, boiled, and centrifuged. The supernatant was used as such, and the precipitate suspended in a volume of pH 6·5 acetate buffer corresponding to that of supernatant removed.

In another experiment, the haemolysate was boiled prior to pH adjustment, and the precipitate suspended in such volumes of pH 6·5 and pH 3·5 buffer as to correspond in concentration to two unboiled haemolysates adjusted to the same pH values. The results of both experiments are shown in Table 9.

TABLE 9.

Activity of boiled systems separated into soluble and insoluble fractions at various pH values.

Conditions: as in Table 7.

Boiled (B) or unboiled (UB).	Precipitate (P) or supernatant (S).	pH.	μl. CO_2 evolved (corrected for autodecomposition). Figures in brackets represent the period, in minutes, after tipping at which the reading was taken.					
UB	—	6·5	25 (5)	45 (10)	63 (15)	113 (30)	139 (45)	153 (60)
B	P	6·5	28 (5)	45 (10)	60 (15)	111 (30)	135 (45)	155 (60)
B	S	6·5	1 (5)	4 (10)	8 (15)	16 (30)	20 (45)	31 (60)
UB	—	6·5	5 (5)	16 (10)	23 (15)	48 (30)		76 (60)
B	P	6·5	10 (5)	29 (10)	30 (15)	55 (30)		77 (60)
UB	—	3·5	62 (5)	154 (10)	213 (15)			
B	P	3·5	97 (5)	220 (10)				

It can be seen clearly that the activity lies almost exclusively in the precipitate. Furthermore, it is of interest to note that the activity of the precipitate suspended at pH 6·5 roughly equals that of the unboiled control whereas the activity of the same precipitate in

pH 3·5 buffer even exceeds that of the control at pH 3·5. This latter finding is further proof of the convertibility of less active (alkaline boiled) to more active (acid boiled) systems.

MISCELLANEOUS RESULTS.

The Action of Cupferron.

It was pointed out earlier that cupferron totally abolished the catalytic effect of inorganic iron and copper on oxalacetate autodecomposition. The influence of this substance on various decarboxylation systems derived from blood was therefore studied. If no decrease in activity occurs, one of the following two conclusions may be drawn: The activity of the system is not due to inorganic iron and/or copper, or, cupferron fails to remove iron and/or copper from the system due to interference of proteins, etc. The former seems to be the more reasonable suggestion, in the absence of any positive evidence for the latter.

The following all gave results which indicate that cupferron has no influence on decarboxylation rate: Haemolysate pH 6·0, haemolysate pH 6·5 (precipitate from boiled fraction), haemolysate pH 6·5 (supernatant from boiled fraction). Since in these typical systems no effect whatever was observed, it is not unreasonable to assume that inorganic Fe and Cu do not play a major rôle in blood decarboxylation under the experimental conditions. Cupferron had no significant influence on the autodecomposition of oxalacetate at pH 6·0–6·5.

Effect of Ferricyanide, Carbon Monoxide and Methylene Blue.

Since it was becoming apparent that the process under examination could well be intimately connected with oxyhaemoglobin, it was thought advisable to test at least a few agents which exert a known effect on the pigment.

Treatment of a haemolysate with excess ferricyanide gave typical, brown methaemoglobin samples. The excess ferricyanide was not removed by dialysis, because control samples in buffer showed that neither it nor ferrocyanide had any significant effect on oxalacetate autodecomposition. The following results may be quoted:

At pH 6·5, the methaemoglobin-containing sample did not differ greatly in activity from an untreated control. If anything, the former was slightly less active. At pH 5·5 a very noticeable reduction in the activity of the treated sample (as compared to the untreated control) was observed. At pH 5·0, the treated sample was again less active than its control. These findings suggest that ferricyanide methaemoglobin is less active than the derivative formed below pH 6·0 (i.e. acid methaemoglobin), and, furthermore, that formation of this more active acid methaemoglobin may not occur after treatment with ferricyanide.

The effect of carbon monoxide was also investigated: At pH 5·5, it was found that samples treated with coal-gas were only slightly less active than the untreated controls. This suggests that the carbon monoxide derivative has approximately the same activity.

Samples treated with small amounts of methylene blue, appear to be slightly more active at pH 6·5 than untreated controls, whereas at pH 5·0 the same significant decreases in activity as found with ferricyanide were observed.

From these studies it appears as though, if oxyhaemoglobin is the agent responsible for decarboxylation at pH 6–7, those derivatives only which are formed by progressively lowering the pH are significantly more active than oxyhaemoglobin itself.

Further studies into the nature of these derivatives are now in progress.

Distribution of Activity in the Acetone Powder.

Following the finding that it was not possible to obtain the full activity of acetone powders in the aqueous extract, the activities of extract and residue were examined at various pH values. The results of a typical sample are summarized in Table 10.

TABLE 10.

Activity of extracts and residues prepared from blood acetone powders.

Conditions: as in Table 7.

Description.	pH.	μl. CO_2 evolved (corrected for autodecomposition) over the following intervals (in minutes) after tipping.					
		5	10	15	20	25	30
Supernatant	6·5	12	15	19	25	28	31
Supernatant	3·5	80	114	129	—	—	—
Residue	6·5	16	24	35	47	55	59
Residue	3·5	144	181	—	—	—	—

It can be seen that, as in the case of all other samples tested, the activity at pH 3·5 is much greater than that at pH 6·5. Furthermore, the residue is more active than the extract. Explanation of this phenomenon in view of the oxyhaemoglobin theory is fairly simple: The extract has been shown to contain oxyhaemoglobin at pH 6·5, which would be converted to the more active acid haematin at pH 3·5. The residue would contain a much higher concentration of some type of haemochromogen, and this, in turn, would be converted to a parahaematin derivative (and possibly a certain amount of acid haematin) at the lower pH value. The greater activity of the residue as compared to that of the extract at both pH values is probably due to the much higher proportion of pigment derivatives in the former.

DISCUSSION.

The results quoted in this paper lead to two main conclusions: firstly, that oxalacetate is extremely sensitive to even low concentrations of certain metallic ions, and secondly, that its decarboxylation in blood is by no means simply an enzymatic process.

The first conclusion has previously been observed. While this work was in progress, Kornberg, Ochoa and Mehler (1948) published data on the catalytic effect of Al^{+++}, and referred to a brief communication by Krebs (1942) who investigated the effect of various salts on oxalacetate decomposition. The results cited here correspond approximately to those of Krebs, but the latter did not describe the O_2 uptakes observed with ferrous iron, nor the unusual behaviour of varying concentrations of ferric iron at different pH values. It remains to be seen just how specific oxalacetate decarboxylations really are, and a whole variety of proteins (and their derivatives) as well as other important biological substances should be investigated. It is also interesting to speculate just how living cells manage to protect this important intermediary from small amounts of Cu^{++} and Fe^{++}/Fe^{+++}. It is quite obvious that much work still remains to be done in these connections.

Several independent experiments suggest that the process in blood is not due to simple inorganic or organic factors. The results with cupferron show that the latter is capable of completely abolishing the catalytic effect of Cu^{++} and Fe^{++} (or Fe^{+++}) on oxalacetate decomposition, whereas it has no effect whatsoever on the activity of systems derived from blood. It is therefore not unreasonable to assume that the process is not due to small amounts of these ions. Then again, dialysis should cause an almost complete loss of activity if the factor responsible for the decarboxylation were a simple, readily diffusible substance. Most convincing of all is the evidence of the considerable activity of the residue from acetone powder extracts, especially when suspended at low pH values. This almost certainly excludes soluble factors, which would to a large extent dissolve in the supernatant during the extraction period. In those systems which are still active after boiling, the activity can be shown to lie almost exclusively in the insoluble portion of the boiled system (indicating an active denatured protein). It therefore seems as though the decarboxylation is associated with protein.

The "enzyme" must therefore satisfy the following conditions:

It must be present in considerable amounts in erythrocytes (since it can be diluted quite extensively without great loss of activity). It appears to be independent of organic co-factors. A reasonable explanation must be found for its very low pH optimum, and for the effect of boiling at low pH values (i.e. *increase* in activity). Any theory regarding the identification of the system must, moreover, account for the finding that under certain conditions boiling causes a *decrease* in activity, whereas in other circumstances increased activities are observed.

From the spectroscopic and preliminary spectrophotometric observations reported earlier in this paper it is quite clear that with lowering of pH, a transition of oxyhaemoglobin to methaemoglobin and finally acid haematin occurs. This is in agreement with the known behaviour of oxyhaemoglobin from other animals.

It is, therefore, not unreasonable to link the decarboxylation directly with oxyhaemoglobin and its derivatives.

The main findings would then be explained as follows:

The actual *in vivo* decarboxylation of oxalacetate in blood is catalysed by oxyhaemoglobin at physiological pH. Whether this catalysis is truly enzymatic, and whether the process is reversible, still remains to be seen. This, therefore, constitutes the first of the two mechanisms stipulated previously (Nossal, 1948b). The "heat lability" referred to at the time can now be accounted for as follows:

The haemochromogen, which no doubt constitutes a considerable portion of the boiled fraction, is less active than the native oxyhaemoglobin at pH 6·5. Furthermore, virtually all the activity of boiled samples has been shown to lie in the particulate fraction, which was not studied fully in earlier experiments.

Below pH 6·0, conversion to methaemoglobin undoubtedly occurs. In accordance with the above theory, it may be concluded that the methaemoglobin produced in the acid medium is more active as a catalyst than the oxyhaemoglobin. At first sight, this might seem to contradict the finding that methaemoglobin produced by treatment with excess ferricyanide is rather *less active* than a corresponding untreated oxyhaemoglobin sample. Actually, according to Lemberg (1948), it is reasonably certain that the methaemoglobins produced in acid and by ferricyanide differ quite appreciably, so that the failure of ferricyanide methaemoglobin to be more active does not constitute an argument against the theory.

Failure of boiling at pH values lower than 6·5 to cause reduction in activity is explained by assuming that the haemichromogens (parahaematins) are no less active than acid methaemoglobin.

Below pH 5·0, the further increase in activity could be accounted for by haematin formation. This has been observed spectroscopically and spectrophotometrically in the more active samples. The *increases* in activity of boiled samples as compared with unboiled controls at pH values below 5·0 would be nicely explained by increased haematin formation, or, at any rate, of a more active haematin-denatured globin complex.

It is of interest to compare this system with others previously described.

The experimental conditions of Breusch (1939) differ rather widely from those employed in this work. Boiling at pH 7·7 would cause conversion of any haemoglobin present to a haemochromogen, and it seems unlikely that this would appear in the filtrate. On the other hand, it is not impossible that the activity could be accounted for by small amounts of active metals, e.g. Cu^{++}.

Evans' (1943) pigeon liver enzyme certainly contained haemoglobin or its derivatives, but it remains to be seen whether the activity can be wholly accounted for by pigment content. Also the activity of pigeon haemolysate would have to be determined. Whether the bacterial enzyme of Krampitz and Werkman (1941) contains a metal-porphyrin protein complex to which its activity could be ascribed, is as yet unknown.

The process here described does not seem to be related to the oxidative decarboxylation catalysed by pig's heart acetone powder extract (Vennesland and Evans, 1944), the active constituent of which has been identified as metmyoglobin (Vennesland, Evans and Francis, 1946). The authors claim that haemoglobin was inactive, but do not state the source or concentration of the pigment used. Since it seems possible that oxyhaemoglobins from various animals will differ in their catalytic activity, this finding cannot be taken as definite proof against the theory proposed above.

Possibly the most marked difference between the decarboxylation in blood and those previously investigated is the apparent independence of the former from metal activators. In spite of rigorous dialysis, the deactivation of dialysed samples was always fairly low, and often negligible. Furthermore, the effects of

both Mn^{++} and Mg^{++} were never even nearly so pronounced as in the bacterial and pigeon liver enzymes. According to Kornberg, Ochoa and Mehler (1948) oxalacetic carboxylase may accelerate the breakdown of an oxalacetate-Mn^{++} complex. In the system discussed here, it seems as though this complex formation may not be necessary, i.e. Mn^{++} does not appear to feature in the process. Alternatively, the active metal may be so firmly attached to the protein that it cannot be removed by dialysis, though this seems less likely. The small activity increases observed in certain experiments could be explained by stipulating a certain amount of metal-substrate complex formation under specific experimental conditions.

Further work in connection with the elucidation of the decarboxylation of oxalacetate in blood is in progress.

SUMMARY.

The influence of pH and of a range of metallic ions on the spontaneous decomposition of oxalacetate has been studied. All the metals except Hg^{++} and Ca^{++} accelerated the process. In the presence of ferrous iron, a simultaneous uptake of O_2 occurs, which amounts to half the CO_2 evolved. Ferric iron shows an increase of activity with dilution in the vicinity of pH 6·0. Lowering of pH greatly accelerates the autodecomposition rate.

A specific oxalacetate decarboxylation has been shown to occur in rabbits' blood. The process is found in whole blood, erythrocytes, erythrocyte haemolysates, plasma and acetone powder extracts of any of these.

Dialysis experiments and certain other considerations suggest that manganese and magnesium do not feature in the system as prominently as in enzymes previously discovered. Thiamine and/or co-carboxylase are not concerned in the decarboxylation.

Progressive lowering of pH from 7·0 to 3·5 causes very marked increases in activity of all the samples tested.

Although under certain conditions boiling may cause almost complete loss of activity, samples boiled at *acid* pH values show either no or only a slight deactivation, and, at low pH values, increases are occasionally observed. The activity of boiled samples is shown to lie almost exclusively in the particulate fraction of the system.

The process is suggested to be *not* due to inorganic iron and/or copper, since cupferron (which completely abolishes the catalytic activity of these two metals) had no effect on the systems derived from blood on which it was tested.

Samples treated with ferricyanide, carbon monoxide and methylene blue were, with one exception, only slightly less active than untreated controls.

The experimental findings are discussed in the light of the suggestion that the decarboxylation is catalysed by, or intimately connected with, oxyhaemoglobin and its derivatives.

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THE METABOLISM OF ERYTHROCYTES

III. IDENTIFICATION OF ONE BLOOD DECARBOXYLATION SYSTEM.

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Although oxalacetate is assuming an ever-increasing importance as a focal point of fat and carbohydrate metabolism, comparatively little is known about the enzymes concerned in its utilization. Oxalacetate itself is notoriously unstable, and its spontaneous decompositon to pyruvate $+CO_2$ is accelerated by a variety of metallic ions and also by H^+ (Krebs, 1942; Nossal, 1948b). Protein-containing extracts from bacteria, plants and animals have been described, but, with one exception, no identification of the systems responsible for the decarboxylation has been cited.

The biological systems specific for decarboxylation of oxalacetate fall into the following chronological order:

A heat-stable factor from liver, muscle, kidney and brain (Breusch, 1939). Oxalacetic decarboxylase from *Micrococcus lysodeikticus*, which requires Mg^{++} but not thiamine or cocarboxylase (Krampitz and Werkman, 1941). A similar enzyme from pigeon liver, requiring Mn^{++} rather than Mg^{++}, and inhibited by malonate (Evans, Vennesland and Slotin, 1943). An oxidative enzyme in a pig's heart preparation, the product of which is malonate, again requiring Mn^{++} (Vennesland and Evans, 1944); this was subsequently identified as metmyoglobin (Vennesland, Evans and Francis, 1946). A plant enzyme similar to that in pigeon liver, and intimately connected with a malic dehydrogenase (Goollub and Vennesland, 1947). Finally, a decarboxylation by blood (Nossal, 1948a and 1948b).

This latter process exhibits a number of very unusual properties in relation to pH and heat, and appears to be independent of metal activators. From a variety of considerations, it was suggested that the catalyst is actually oxyhaemoglobin (and certain of its derivatives), or intimately connected therewith.

This communication represents the results of further manometric estimations of activity, and simultaneous spectrophotometric examination, in the light of the above theory.

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[2] Working under a grant from the Sheridan Research Fund.

METHODS.

Treatment of the materials was essentially similar to that previously described (Nossal, 1948b). To make all the experiments as mutually comparable as possible, the cells were always lysed with sufficient dilute NH_4OH to make the sample up to twice the original blood volume. Furthermore, pH adjustments were made by diluting one volume of haemolysate with an equal volume of the appropriate 2M acetate buffers. All starting materials were, therefore, equivalent to blood diluted to four times its volume, unless otherwise stated.

Throughout this work, 2·0 ml. of the catalyst system were used in each Warburg cup, the total volume of fluid per cup being maintained at 3·0 ml. Oxalacetate concentration was *ca.* 10–2M throughout.

Measurement of CO_2 evolution was carried out with standard Warburg equipment at 38° C. Generally speaking, readings were taken 10 minutes after immersion in the bath, and the experiments commenced by tipping 20 minutes after immersion. The experimental period was 30 minutes, unless otherwise stated, readings being taken at five-minute intervals.

It was soon obvious that at any pH below 6·5, haemolysates would contain mixtures of oxyhaemoglobin and methaemoglobin, and also methaemoglobin and acid haematin. Such mixtures are known to be notoriously unstable, and yet we felt that it was imperative to know the relative proportions of these substances at the commencement (and occasionally at the conclusion) of the manometric experiments. This was to be attempted by spectrophotometric analysis. Obviously, it was almost impossible to achieve this purpose by the use of any ordinary instrument, since absorption curves of the samples about to be tested for catalytic activity would have taken too long to record with a manual instrument.

Fortunately, a General Electric automatically recording universal spectrophotometer was made available to us for this work. With this instrument it becomes possible to obtain a complete transmission spectrum in 3–6 minutes for any given sample. Thus, we could synchronize the commencement of manometric experiments and the dilution and spectrophotometric examination of catalytic systems very accurately.

All pH measurements were made on glass-electrode systems. Occasionally the pH after completion of the experiments was also measured, but no significant shifts were observed.

The spectrophotometer was calibrated against oxyhaemoglobin, methaemoglobin and haematin samples of known concentration.

Calculation of Results.

All manometric activity experiments are expressed as the rate of CO_2 evolution due to oxalacetate decarboxylation.

Mixtures of Haemoglobin and Methaemoglobin.

Pigment contents of the reaction mixtures were computed from the recorded transmissions at 5,400 Å and 5,000 Å, the results being expressed as gm./ml. of oxyhaemoglobin and methaemoglobin, using the relationship

$$\tau = k10^{-\sum_{i=1}^{n} a_{\lambda_i} O_i}$$

At best the computed values are approximations since opacity frequently occurred in the samples after prolonged incubation at pH 5. This appears due to precipitation of the pigment, the tendency for such precipitation varying from animal to animal, and seems related to idiosyncrasies of the isoelectric point of denatured haemoglobin derived from the different bloods.

RESULTS.

Behaviour of Activity and of Oxyhaemoglobin with Progressive Lowering of pH.

The increase in the activity exhibited by any haemolysate with falling pH values has been observed previously (Nossal, 1948b). Described below, however, is an experiment, in which the whole pH range from 6·5 to 3·5 was examined *simultaneously* on the one blood sample. This was made possible by the use of two identical Warburg baths and 24 manometer sets.

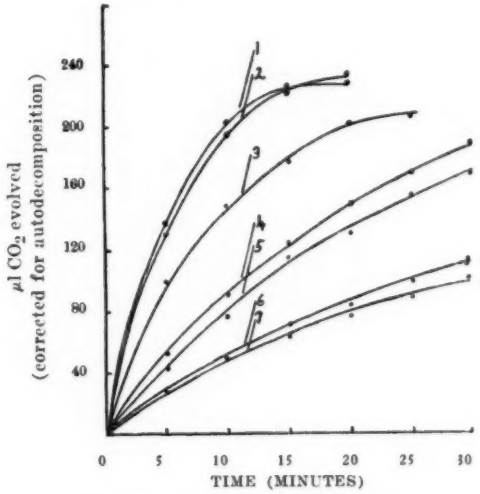

Fig. 1. Activity of rabbit haemolysate at varying pH values:

Curve 1, pH 3·5; Curve 2, pH 4·0; Curve 3, pH 4·5; Curve 4, pH 5·0; Curve 5, pH 5·5; Curve 6, pH 6·0; Curve 7, pH 6·5.

Samples were examined spectrophotometrically before and after the experimental incubation. The manometric activities and spectrophotometric findings are shown in Figs. 1 and 2 (and Table 1).

The following observations concerning these findings should be recorded: Six of the seven activity curves fall into three distinct groups when examined quantitatively:

The activities for the pH pairs 6·5 and 6·0, 5·5 and 5·0, and 4·0 and 3·5 differ from each other much less than each differs from that lying to the other side of it (e.g. 6·0 from 5·5, and 4·0 from 4·5). At the same time, both samples of pH 6·5 and 6·0 contain initially *and* finally largely oxyhaemoglobin, those of pH 5·5 and 5·0 contain finally larger amounts of methaemoglobin, and those at pH 4·0 and 3·5 both contain mainly haematin.

The activity of the pH 4·5 sample lies more evenly between the 5·0 and 4·0 samples, because it constitutes a mixture of both methaemoglobin and haematin.

Fig. 2 is included to record the changes which rabbit oxyhaemoglobin undergoes during incubation at 38° C. at various hydrogen ion concentrations.

In both A and B the presence of isosbestic points at about 410, 460, 520 and 590 millimicrons indicates the development of an equilibrium mixture in the solutions as the pH is lowered from pH 6·5 to 5·0 (curves 1, 2 and 3). Haemoglobin is converted to methaemoglobin, with appearance of maxima at 500 and 630 millimicrons increasing rapidly as the pH becomes lower; the curve for pH 6·0 has been omitted from B but falls between 1 and 2 (see Table 1).

At pH 4·5 the denatured haemoglobin tends to precipitate so that curves 5 in A and B are displaced by cloudiness in the solutions; the maxima at 530 and 630 millimicrons in B5 indicate that haematin is produced at pH 4·5 but more slowly than at pH 4·0 or 3·5.

Curves 4 in A and B are virtually identical so that conversion to haematin at pH 4·0 and 3·5 must be complete after 20 minutes' incubation of the haemolysate at 38° C.

TABLE 1.

Behaviour of rabbit haemolysate during incubation at 38° C. at various pH values.

Samples were examined spectrophotometrically before immersion in the bath, at the commencement of the experiment (tipping of substrate in the Warburg cups) (20 minutes after immersion) and at the conclusion of the experiment (30 minutes later).

All values represent gm. of pigment/ml. in the cups. All cups contain 2·0 ml. haemolysate (adjusted to the appropriate pH as usual), 0·8 ml. buffer and 0·2 ml. oxalacetate.

	Oxyhaemoglobin			Methaemoglobin			Acid Haematin		
pH	Initial	Tipping	Final	Initial	Tipping	Final	Initial	Tipping	Final
6·5	0·018	0·018	0·017	—	trace only	0·001	—	—	—
6·0	0·018	0·018	0·017	—	trace only	0·003	—	—	—
5·5	0·018	0·017	0·013	trace only	0·002	0·006	—	—	—
5·0	0·018	0·0145	0·01	5 p.c. of HbO_2	0·0045	0·01	—	—	—
4·5	0·018	cloudy (still mainly oxy-haemoglobin)	—	5 p.c. of HbO_2	cloudy, increase in methaemoglobin	—	—	—	cloudy (mainly acid haematin)
4·0	0·014	—	—	0·002	—	—	—	$0·07\times10^{-2}$	$0·08\times10^{-2}$
3·5	traces	—	—	traces	—	—	mainly acid haematin	$0·07\times10^{-2}$	$0·08\times10^{-2}$

TABLE 2.

Activity and behaviour of horse oxyhaemoglobin and pigeon blood haemolysate at various pH values.

Conditions: Each cup contains 2·0 ml. of the test system, 0·8 ml. buffer and 0·2 ml. oxalacetate (side-bulb) to a final concentration of approximately 10^{-2}M. T = 38° C. Readings taken at five-minute intervals. All figures corrected for autodecomposition rate. Experimental period: 30 minutes.

System	pH	Rate of CO_2 evolution/5 mins. (corrected) in μl. for 30 mins.						Spectrophotometric examination.*					
								Haemoglobin		Methaemoglobin		Haematin	
		1	2	3	4	5	6	Initial	Final	Initial	Final	Initial	Final
Horse oxyhaemoglobin	6·5	15	27	36	41	48	53	0·007	0·005	0·007	0·008	—	—
Horse oxyhaemoglobin	5·0	32	59	78	93	103	114	0·002	0·0005	0·011	0·013	—	—
Horse oxyhaemoglobin	3·5	98	171	189	—	—	—	—	—	—	—	$0·09\times10^{-2}$	$0·09\times10^{-2}$
Pigeon blood haemolysate	6·5	33	51	65	75	92	—	·015		—		—	
Pigeon blood haemolysate	5·0	42	71	96	112	133	—	0·002		0·011		—	
Pigeon blood haemolysate	3·5	122	184	201	—	—	—	—		—		$·06\times10^{-2}$	

* Figures represent gm. pigment/ml. in the experimental cup. Oxyhaemoglobin was examined prior and subsequent to the activity measurement, the pigeon haemolysate only before the experiment.

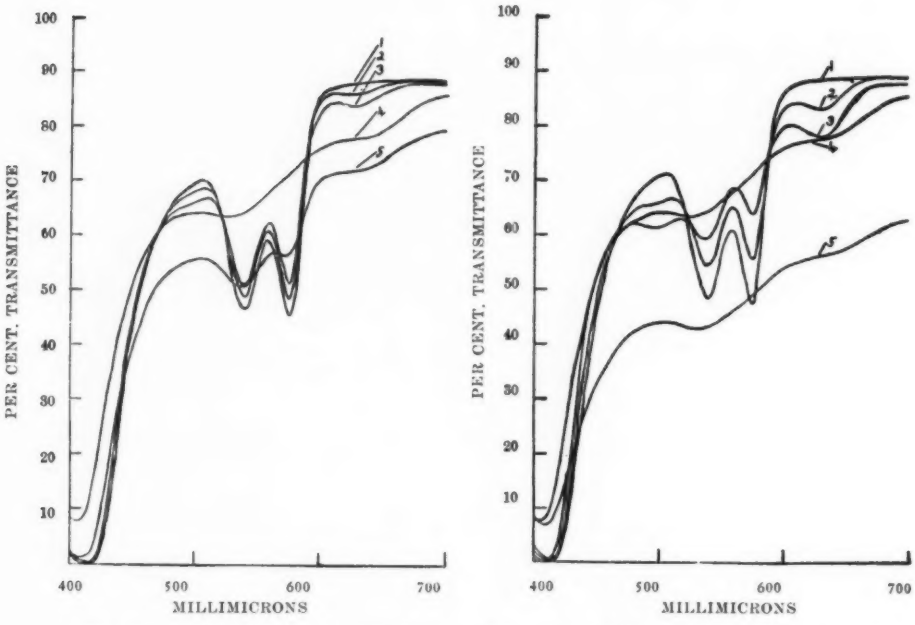

Fig. 2. Spectrophotometric examination of incubated haemolysates at varying pH values. (Pigment contents as in Table 1.)

A. Withdrawn and examined just prior to tipping, i.e. after 20 minutes incubation at 38° C.

1, pH 6·5 and 6·0; 2, pH 5·5; 3, pH 5·0; 4, pH 4·0 and 3·5; 5, pH 4·5.

B. Withdrawn and examined at completion of run, i.e. after 50 minutes incubation at 38° C.

1, pH 6·5 (pH 6·0 omitted); 2, pH 5·5; 3, pH 5·0; 4, pH 4·0 and 3·5; 5, pH 4·5.

The Behaviour and Activity of Purified (Crystalline) Oxyhaemoglobin and of Pigeon Haemolysate at Various pH values.

Further proof of our hypothesis was furnished by the experiments with crystalline horse oxyhaemoglobin (generously supplied by Mr. E. Foulkes). A typical activity experiment is shown in Table 2.[3]

It can be seen that the behaviour of this sample with decreasing pH is identical with that of the other blood constituents previously described.

An avian blood was examined to eliminate the possibility that the process is specific for mammalian bloods. Once again, as seen from Table 2, the same gradation of activity with pH variation was observed.

It appears, therefore, that the connection between this oxalacetate decarboxylation and oxyhaemoglobin and its derivatives is fairly firmly established. It remains to be shown

[3] It can be seen that the sample contained a high proportion of initial methaemoglobin. However, the behaviour in all other respects is exactly the same as that of fresh haemolysates.

whether the process is reversible, what is its exact relation to other known decarboxylases, and also, whether the bulk of the activity is centred around the protein or the prosthetic group.

Examination of a Pigeon Liver Acetone Powder Extract.

We prepared these extracts from pigeons exactly as described by Evans *et. al.* (1943), with the exception that our preparations were a little less concentrated.

The following results, not previously observed, should be recorded: The behaviour of the pigeon liver acetone powder is similar to that of systems prepared from blood, nor does the powder show much greater activity at the temperature employed. In both cases, the extract itself is more active at pH 3·5 than at higher values; in both cases, also, the centrifuged residues suspended in an equivalent volume of buffer were more active than the supernatant solution, especially at pH 3·5 (Table 3).

TABLE 3.

Activity of pigeon liver acetone powder and rabbits' blood acetone powder at pH 6·5 and 3·5.
Conditions: As in Table 2.

System *	pH	μl. CO_2 evolved (corrected) over the following intervals (in minutes) after tipping:					
		5	10	15	20	25	30
Pigeon liver APX (supernatant)	5·0	27	40	58	69	79	88
Pigeon liver APX (residue)	5·0	40	75	104	124	142	149
Pigeon liver APX (supernatant)	3·5	40	83	116	131	—	—
Pigeon liver APX (residue)	3·5	117	174	194	—	—	—
Rabbits' blood APX (supernatant)	6·5	12	15	17	25	28	32
Rabbits' blood APX (residue)	6·5	15	24	35	47	55	63
Rabbits' blood APX (supernatant)	3·5	79	114	129	—	—	—
Rabbits' blood APX (residue)	3·5	144	181	—	—	—	—

* APX = aqueous acetone powder extract, supernatant = soluble fraction adjusted to the appropriate pH.

Residue = particulate fraction suspended in the appropriate amount of buffer. All concentrations are comparable.

Spectrophotometric examination of the pigeon liver extract disclosed the presence of blood pigment. Because our preparations were cloudy, it was not possible to determine blood pigment quantitatively by this means. It appears, however, as though the content of pigment (oxyhaemoglobin, methaemoglobin and haematin) does not account for the total activity, unless pigeon oxyhaemoglobin is more active than that of the rabbit. At any rate, it must be obvious that the pigeon liver system requires further, more detailed investigation. The occurrence of appreciable amounts of blood pigment and the similarity in behaviour towards pH to that of blood systems seem to indicate the presence of a factor common to both. Also, it should be noted that boiling of an aqueous acetone powder extract of pigeon liver prior to pH adjustment caused a considerable reduction in activity, but that boiling of the residue in pH 3·5 buffer gave a fairly active extract, as was the case with blood systems (see Table 4).

TABLE 4.

Effect of boiling on pigeon liver acetone powder activity.

Conditions: As in Table 3. Period: 30 minutes.

System	pH	CO_2 evolution (μl.) (as in the last Table)					
		1	2	3	4	5	6
Buffer only	5·0	17	32	42	58	70	84
Pigeon liver APX (supernatant)	5·0	88	162	196	211	225	230
Pigeon liver APX (boiled before pH adjustment)	5·0	38	68	91	118	139	161
Buffer only	3·5	39	69	96	121	147	166
Pigeon liver APX (supernatant)	3·5	79	152	212	252	278	294
Pigeon liver APX (residue boiled in pH 3·5 buffer)	3·5	60	123	179	226	263	276

Critical Examination of Plasma Activity as Related to Oxyhaemoglobin Content.

It has been previously recorded (Nossal, 1948b) that many of the plasma samples investigated contained visible amounts of oxyhaemoglobin. Partly for that reason, dilution experiments with red-cell haemolysates were carried out to determine activity with decreasing pigment concentration. In all cases, forty-fold dilution caused very marked lowering of activity, as might be expected.

The activities of plasma samples were, therefore, re-examined with simultaneous spectrophotometric estimations of the amount of pigment present. Table 5, a record of a typical experiment indicates a low pigment content, whereas the activity is quite considerable.

TABLE 5.

Activity and pigment content of plasma samples under varying pH conditions.

Conditions: As in Table 2. Plasma samples adjusted to the appropriate pH by addition of an equal volume of buffer. Spectrophotometric measurements were made before commencement of the experiment.

pH	CO_2 evolution in μl. (corrected) over 30 minutes (as in Table 3)						Pigment content (as gm./ml. of the system in the experimental cups)			Activity of correspondingly diluted haemolysates as μl. CO_2 (corrected)*
	1	2	3	4	5	6	Oxyhaemoglobin.	Methaemoglobin.	Haematin.	
6·5	30	60	79	104	122	133	0·001	0·0011	—	10–22
6·5	26	57	76	101	119	132				
5·0	32	64	85	109	126	148	—	0·0038	—	19–43
3·5	79	141	168	—	—	—	—	—	$0·009 \times 10^{-2}$	45–95
3·5	89	162	187	—	—	—				

* These activities were determined by dilution of the usual 1:4 haemolysates with buffers. The lower figures of the range represent the activity of samples of ten-fold dilution, the higher figures are activities of five-fold dilution. For pH 6·5 and 5·0, the figures represent total CO_2/30 minutes, whereas for pH 3·5 the period is 15 minutes.

This observation, together with the results relating to acetone powder extracts of pigeon liver, makes it difficult to assume that the total activity of plasma samples can be accounted for by blood pigments. If this were so it would be necessary to stipulate that, in plasma, the pigment is much more active. This would surely mean the presence of some coenzyme or activator. We do not consider this correct, for the following reasons: Dialysis of plasma samples does not cause a sufficiently significant deactivation (Nossal, 1948b), which rules out diffusible cofactors and activators. Furthermore, we have tested the effect of cupferron, previously shown completely to inhibit the catalytic effects of inorganic iron and copper, on oxalacetate decarboxylation on plasma samples at pH 6·5 and 5·0 without observing significant decreases in their activity. It seems, therefore, that plasma activity also can not be ascribed to these metals. Hence, other proteins in plasma may also feature in the decarboxylation, although in samples contaminated with oxyhaemoglobin the latter undoubtedly plays some part.

It is interesting to note that the difference in activity between the samples at pH 6·5 and pH 5·0 is much less pronounced than in the haemolysate, and that the activity increase of the pH 3·5 sample is greater.

Examination of Other Iron-Porphyrin-Proteins.

We made preliminary tests on cytochrome *c* and catalase to see whether the decarboxylation was a general property of haem proteins.

Two purified solutions of cytochrome *c* were both found to be virtually inactive, when tested at pH 6·5 and 3·5. Catalase, prepared by dilution of a crystalline catalase sample with the appropriate buffers, showed quite an appreciable activity (Table 6).

TABLE 6.

Activity of cytochrome c and catalase.

Conditions: As in Table 2.

System	pH	μl. CO_2 evolved (conditions as for preceding Tables)					
		1	2	3	4	5	6
Cytochrome *c*	6·5	12	12	17	17	20	21
Cytochrome *c*	3·5	11	19	30	35	40	45
Catalase	6·5	23	42	67	86	100	111
Catalase	3·5	33	59	82	97	96	109

It is interesting to note that the activity of catalase at pH 3·5 does not greatly exceed that at pH 6·5. This might be due to the greater stability of the protein complex towards acidification.

Since the absolute concentrations of both these preparations were not known, it would be rash to draw any conclusions. Furthermore, the catalase might well contain appreciable amounts of iron and, unfortunately, it was not possible to test the effect of cupferron on it at the time.

Experiments with Haematin-containing Samples.

When a haemolysate brought to pH 3·5 in the usual manner is treated with alkali, precipitation of the denatured protein occurs. At pH 5·0, little precipitation was observed in our samples, whereas precipitation at pH 5·5 was quite extensive, and was apparently complete at pH 6·5.

TABLE 7.

Behaviour of haematin-containing haemolysates at various pH values.

Conditions: As in Table 2. Haemolysate first brought to pH 3·5, and then treated with N NaOH till requisite pH value was obtained. All samples were subsequently diluted to the same volume with the appropriate buffer to equalize pigment concentration.

pH	Comments	μl. CO_2 formed (corrected over 5 minutely intervals as previously)					
		1	2	3	4	5	6
3·5	Original sample	67	113	134	—	—	—
4·5	No precipitation	65	135	149	—	—	—
3·5	Original sample	105	146	175	—	—	—
5·0	Turbidity	70	103	146	175	190	202
5·5	Some precipitation	59	82	119	147	164	176
3·5	Original sample	75	123	154	—	—	—
6·5	Precipitation considerable	2	5	10	12	16	20

Several samples were tested for activity, and some representative results are quoted in Table 7. It can be seen that at pH 4·5 the sample is quite as active as the original sample at pH 3·5 (diluted to bring it to the same volume as the former), the sample at 5·0 is still reasonably active, whereas that at pH 6·5 has lost almost all its activity. Furthermore, examination of the pH 5·5 sample (which was only partly precipitated) showed that the greater part of the activity lay in the faintly-coloured supernatant solution (colloidal haematin, etc.), the heavy brown precipitate in pH 5·5 buffer being rather less active.

It appears, therefore, that the high activity of the systems at pH 3·5 must be largely due to substances in true or in colloidal solution.

Attempts were made to obtain such solutions by trying to dissolve purified acid haematin (prepared from horse-blood in the usual manner) under various conditions. It is well known that this substance is readily soluble in alkali, but precipitates invariably on the acid side of neutrality. A 0·1 p.c. solution at pH 7·0 in phosphate buffer seemed the strongest solution at that pH. This was almost entirely inactive, though this, owing to the low concentrations, provides no proof of the catalytic inactivity of haematin.

More concentrated solutions were prepared, and gum acacia as well as egg albumin tried as aids in the formation of a colloidal suspension at acid pH. Although precipitation still occurred, a number of samples were tested for activity. Surprisingly enough, the mixtures, although their activity never exceeded 90 μl. CO_2/30 minutes above the decomposition rate, seemed more active at pH 3·5 (when precipitation was more pronounced) than at 6·5.

However, until it is possible to obtain a truly colloidal solution of haematin at pH 4·5–3·5, the proof of its catalysis of oxalacetate decarboxylation cannot be claimed.

DISCUSSION.

The evidence to hand concerning the decarboxylation of oxalacetate by blood may now be summarized as follows:

The process becomes progressively more active with decreasing pH values. Simultaneously, a conversion of oxyhaemoglobin to methaemoglobin and finally acid haematin is proved to occur by spectrophotometric examination. The pH

effect is not confined to rabbits' blood, but a parallel activity increase is shown by pigeons' blood. Furthermore, crystalline horse oxyhaemoglobin shows a similar behaviour in respect to hydrogen ion concentration.

Certain results of experiments on the autodecomposition of oxalacetate could be taken to suggest that at the pH values 5·0 and 3·5 the molecule is particularly reactive. Initially it could have been argued that the *same* factor was responsible for the decarboxylation at all pH values, and that the increases in the speed of the process were simply due to increased lability of the substrate. But, since pure oxyhaemoglobin gives almost identical results with those obtained with corpuscular haemolysates, it seems somewhat unlikely to assume that the conversion of oxyhaemoglobin to methaemoglobin and finally acid haematin is merely coincidental with the observed increases in activity. Furthermore, the finding that a sample initially brought to pH 3·5 (to cause haematin formation) and then back to pH 4·5 (at which value little precipitation was observed) is as active as a corresponding pH 3·5 sample, rather suggests the activity of some factor other than hydrogen ion concentration.

From the observations made with Evans' pigeon liver enzyme it seems that this factor is rather similar to that operative in blood. The same increases are observed as the pH is lowered; boiling without pH adjustment causes a reduction in activity, which is in many ways like that encountered in blood (though possibly somewhat more marked). Both for pigeon liver and blood considerable activity is found in systems containing the *residue* from acetone powder extracts suspended in pH 3·5 buffer.

Our findings lend themselves to three interpretations. The first is that oxyhaemoglobin (and its derivatives) is the agent responsible for the decarboxylation. This, as yet, fails to account for the activity of plasma and of pigeon liver extract. The second would be to assume that the actual catalyst is located in the plasma. It becomes necessary, then, to account for the high activity of cell haemolysates (which could only be slightly contaminated with plasma) and of the purified oxyhaemoglobin, by assuming contamination of both with the plasma catalyst. Since it is almost certain that the factor is not simply an enzyme, it is difficult to see how both oxyhaemoglobin and cell haemolysate could be contaminated to such an extent as to possess the high activities which are actually found.

Thirdly, it is possible that both cells and plasma contain active agents. That these would be identical is suggested by the very similar behaviour of both systems towards pH and heat. On the other hand, it could be argued that, in the cell, the agent was oxyhaemoglobin, whereas in the plasma it has been shown not to be. At present our results favour the theory that blood pigments have a catalytic rôle in oxalacetate decarboxylation, but that this finding does not account for the activities of plasma and of pigeon liver extract.

A catalytic rôle in oxalacetate decarboxylation having thus been ascribed

to oxyhaemoglobin and its derivatives, it is interesting to speculate whether this catalysis is due to the prosthetic group or the protein molecule.

Kornberg, Ochoa and Mehler (1948) have proved the formation of complexes of great instability between aluminium ions and oxalsuccinic and oxalacetic acids. In view of their results, it is quite possible that similar complexes could be formed between haem (or haematin) and oxalacetate in blood.

On the other hand, certain amino groupings (such as aniline citrate) quantitatively decompose oxalacetate. It is therefore not impossible that the free $-NH_2$ groups of the protein moiety of oxyhaemoglobin might feature in the process.

In the first case, increase in activity with lowering pH would be explained by (a) conversion of the haem group of oxyhaemoglobin to the haematin of methaemoglobin and (b) liberation of free haematin at pH values below 4·5. In the second case, the progressive activity increases could be explained by stipulating a progressive liberation of $-NH_2$ groups by acid denaturation.

Our results up-to-date do not conflict with either hypothesis. The boiled samples all contain large amounts of denatured globin as well as haemochromogens and parahaematins. The same applies to the residues of acetone powder extracts. Actually, the investigation of plasma and of pigeon liver extracts suggests that in these two systems the activity cannot be fully explained in terms of blood pigment content, since the latter is comparatively low. It is certain that other factors feature in the decarboxylation there.

Further work towards the elucidation of the *mechanism* of this new decarboxylation is in progress.

SUMMARY.

The behaviour of the oxalacetate decarboxylation by blood systems was again investigated in relation to pH. Previous findings (i.e. progressive increases in activity with decreasing pH values) are confirmed. Simultaneously, a transition from oxyhaemoglobin to methaemoglobin and finally to acid haematin is shown to occur, by means of a rapid automatically recording spectrophotometer.

Crystalline horse oxyhaemoglobin and pigeon erythrocyte haemolysate are shown to behave in exactly the same manner when their decarboxylating powers are examined at varying pH values, so that the process in haemolysed erythrocytes is ascribed to haemoglobin and its derivatives.

Although the activities of plasma and acetone powder extracts of pigeon liver do not seem to be totally accounted for by their blood pigment content, many points of similarity to blood are shown which suggest that the mechanisms are not unrelated.

Preliminary experiments with other haem proteins, and attempts to prepare a colloidal solution of crystalline haematin at acid pH to determine its activity, are reported.

The possible mechanisms of the decarboxylation by blood systems are discussed.

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